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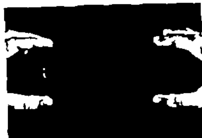
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[illegible], R. J Clin Microbiol 6 (1977) p. 23–26

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[illegible], T et al. [illegible] 159 (1974) p. 191

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[illegible], H. Acta Path et Microbiol Scand 84 (1976) p. 79–84

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[illegible], R. J Clin Microbiol 6 (1977) p. 23–26

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LIGHT AND ELECTRON MICROSCOPY ON THE SPORULATION OF THE OOCYSTS OF *EIMERIA BRUNETTI*

I Development of the Zygote and Formation of the Sporoblasts

D J P FERGUSON[1,2]* A BIRCH ANDERSEN[2] W M HUTCHISON[3] and J. CHR. SIIM[1]

FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut, Copenhagen, Denmark, and Department of Biology[3] University of Strathclyde, Glasgow Scotland

Ferguson, D J P Birch-Andersen, A., Hutchison, W M. & Siim, J Chr Light and electron microscopy on the sporulation of the oocysts of *Eimeria brunetti* I Development of the zygote and formation of the sporoblasts Acta path microbiol scand Sect. B 86 1–11 1978

The initial stages of sporulation in oocysts of *Eimeria brunetti* were examined in samples sporulated at 27°C for 0 12 and 24 hours The initial zygote was found to be roughly spherical and to contain a number of polysaccharide granules which were congregated in one region of the organism. The cytoplasm also contained some strands of rough endoplasmic reticulum together with a number of mitochondria, some Golgi bodies, and some electron translucent vacuoles The nucleus was large, with amorphous nucleoplasm and a nucleolus The cytoplasmic mass of the zygote was limited by a single unit membrane which possessed some micropores After initiation of the sporulation, the metabolic activity of the organism appeared to increase as evidenced by the augmentation in the cytoplasm of the amounts of rough endoplasmic reticulum, number of Golgi bodies, and the appearance of polyribosomes However at this stage the presence of large spherical dense bodies (anlagen of the refractile bodies of the sporozoites) constituted the most obvious change in the cytoplasm of the organism After nuclear division the daughter nuclei were situated well separated in the cytoplasm and the polysaccharide granules were evenly distributed throughout the cytoplasm of the zygote. Eventually four sporoblasts were formed by invaginations of the limiting membrane Each sporoblast was limited by unit membrane and contained a nucleus, and the same cytoplasmic organelles as found in the zygote The development of the sporoblast was initially accompanied by the appearance of a second limiting membrane

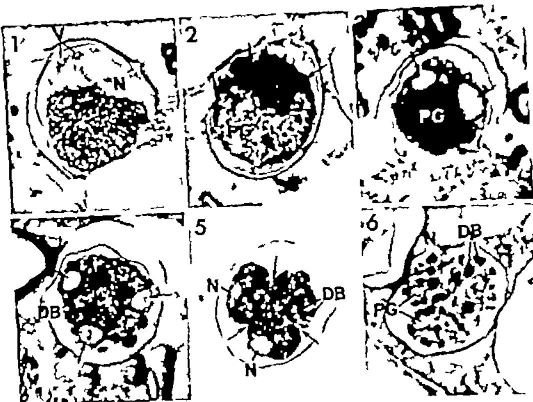
Key words *Eimeria brunetti* sporulation, zygote development, sporoblast formation; ultrastructure

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Received 25 ii 77 Accepted 9 viii 77

The oocysts of parasites belonging to the family *Eimeriidae* undergo development (sporulation) in the external environment. At present the ultrastructural aspects of the sporulation have not been studied because of difficulties in preparing the oocysts for examination by electron microscopy Recently a technique has been described for preparing thin sections of oocysts for observation in the electron microscope (1) We have briefly

* Work initiated while a Wellcome Trust Travelling Research Fellow and completed as Danish Medical Research Council Fellow



Figures 1–6 are light micrographs and Figures 7–26 are electron micrographs of sections and illustrate the development of the zygote and formation of the sporoblasts within oocysts of *E. brunetti*

A double bar (=) on a figure represents 1 μm and a single bar (–) 100 nm.

The following abbreviations are used throughout AM = amorphous body CE = centriole; DB = dense body (precursor of the refractile body); ER = rough endoplasmic reticulum, EV = electron translucent vacuole, G = Golgi body LM = limiting membrane, MI = mitochondrion, MP = micropore, N = nucleus; NM = nuclear membrane, NP nuclear pole. NU = Nucleolus, OW = oocyst wall, P = possible phagocytic vacuole; PG = polysaccharide granule, R = ribosome, UM = unit membrane, V = vacuole

Fig 1 A section through an unsporulated oocyst (0 hours) The polysaccharide granules are congregated at one side of the zygote The nucleus and a number of vacuoles can also be seen 2,000 ×

Fig 2 A section through a sporulating oocyst. A number of dense bodies (arrows) are present in addition to the polysaccharide granules, vacuoles and the nucleus (cf Fig 1) 2,000 ×

Fig 3 In this section nuclear division has occurred and two nuclei are visible (arrows) 2000 ×

Fig 4 A section through an organism in which three nuclei (arrows), well separated from each other are included in the section Note the even distribution of dense bodies and polysaccharide granules 2000 ×

Fig 5 In this section the cytoplasmic mass has started to divide and form the sporoblasts The points of invagination are marked by arrows 2000 ×

Fig 6 Three of the four newly formed sporoblasts are included in this section Each sporoblast contains a nucleus, a number of dense bodies, and polysaccharide granules 2000 ×

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phagocytic vacuoles (Figs 14 and 15). In other parts of the surface of the zygote, electron translucent vacuoles and amorphous membrane bound structures appeared to be ejected from the cytoplasm (Fig.

In the cytoplasm the amount of rough endoplasmic reticulum and the number and apparent activity of the Golgi bodies were increased (Figs 13 17 and 19). The number of free ribosomes also increased and polyribosomes were noticed. A number of

reported on a few results obtained using this technique to study the developing oocysts of *Eimeria brunetti* (12). In the present paper details of the structural changes observed during the initiation of sporulation of the zygote of *E brunetti* will be given. In addition the changes accompanying the formation of the sporoblasts will be reported and the results will be compared with those reported for other members of the Sporozoa.

MATERIALS AND METHODS

The oocysts of *E brunetti* used in this study were concentrated from chicken faeces and stored at 4 C.

Prior to processing for electron microscopy the oocysts were allowed to sporulate at approximately 27° C for 0 12 24 36 or 48 hours. Each of the samples was then processed by the technique described by *Birch Andersen et al* (1) This technique can be summarized as follows Oocysts were pre-embedded in cross linked bovine serum albumin (BSA) and sectioned with a cryostat after rapid freezing in liquid nitrogen. The cryostat sections were allowed to thaw in Karnovsky s fixative, re-embedded in BSA, and then post fixed in osmium tetroxide before dehydration and final embedding in Vestopal W Light microscopy was carried out on 1 µm thick sections stained with toluidine blue and photomicrographs were obtained with a Carl Zeiss photomicroscope II using Agfapan 25 professional film. Thin sections were examined in the electron microscope after staining with magnesium uranyl acetate and lead citrate. The results in this paper are based on the examination of more than 700 electron micrographs.

RESULTS

Light Microscopy

The morphology of the oocysts containing the zygote, prior to the initiation of sporulation, was examined in material processed directly from storage at 4° C (sporulation time: 0 hours). Progressive developmental changes were observed in samples which had been allowed to sporulate for increasing periods of time. After 12 hours, a few of the oocysts had reached the sporoblast stage, but by 24 hours more than 50 per cent had developed to this stage. Development was apparently not synchronized and even after 48 hours of sporulation it was possible to observe oocysts which contained well preserved but still undivided zygotes.

In sections of the 0 hour material the zygote appeared spherical Polysaccharide granules were observed congregated together in one half of the cytoplasm of the organism The nucleus was situated in the area of cytoplasm free of polysaccharide granules and a number of vacuoles were present at the periphery of the zygote (Fig 1).

After 12 hours of sporulation the most marke[d] difference noted in the toluidine blue staine[d] material was the appearance of a number of den[se] spherical bodies in the cytoplasm of the zygote (Fi[g] 2). In organisms in which nuclear divisions ha[d] occurred the nuclei were generally well separate[d] and mainly found close to the periphery of th[e] organism (Fig 3). At this stage of development th[e] spatial relationship between the intracellular inclu[-]sions had changed so that now the dense bodies an[d] the polysaccharide granules were evenly distribute[d] thoughout the cytoplasm (Fig 4)

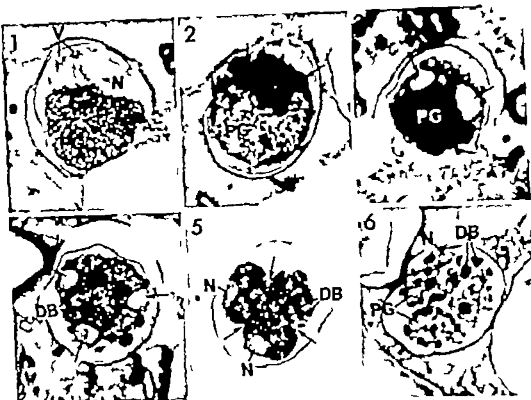
In sections prepared from material obtained aft[er] 24 hours of sporulation it was possible to find [a] number of organisms in which the zygote ha[d] divided and formed four sporoblasts (Fig 6) [It] would appear that the four sporoblasts were forme[d] simultaneously (Fig. 5).

The sporoblasts were spherical initially bu[t] became more ellipsoidal in shape and contained [a] nucleus, some polysaccharide granules, some dens[e] bodies and some vacuoles (Fig. 6).

Electron Microscopy

In thin sections the zygote of unsporulate[d] oocysts (0 hour material) was found to be limited b[y] a single unit membrane (Fig 8). The outline of th[e] organism was irregular with a number of invagina[-]tions of the limiting membrane. Typical inactiv[e] micropores (13) were present on this membran[e] The cytoplasm of the zygote contained a number [of] polysaccharide granules which, as previously obser[-]ved by light microscopy were densely packed in[to] one portion of the cytoplasmic mass (Fig. 7). [A] number of electron translucent vacuoles wer[e] present in association with the polysacchari[de] granules The translucent vacuoles were als[o] observed close to the periphery of the organis[m] together with a number of mitochondria Som[e] rough endoplasmic reticulum and a few Gol[gi] bodies were observed in the portion of cytoplas[m] which was free from polysaccharide granules (Fi[g] 7) The nucleus was present in the same regio[n] where it appeared as a large diffuse structure, wit[h] an indistinct limiting membrane and lacking dens[e] patches of chromatin (Figs 7 and 9). In a few cas[es] a nucleolus was observed.

The ultrastructural changes occurring within th[e] zygote prior to its division into sporoblasts wer[e] examined in material which had sporulated for 1[2] and 24 hours. The shape of the cytoplasmic mass o[f] the zygote was found to remain unchanged (Fig 13), although certain organisms did show a[n] increase in the number and extent of the invagina[-]tions of their limiting membranes (Fig 14). I[t] appeared that these invaginations could form



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In the cytoplasm the amount of rough endoplasmic reticulum and the number and apparent activity of the Golgi bodies were increased (Figs 13, 17 and 19). The number of free ribosomes also increased and polyribosomes were noticed. A number of

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In sections of the 0 hour material the zygote appeared spherical Polysaccharide granules were observed congregated together in one half of the cytoplasm of the organism. The nucleus was situated in the area of cytoplasm free of polysaccharide granules and a number of vacuoles were present at the periphery of the zygote (Fig 1).

After 12 hours of sporulation the most marked difference noted in the toluidine blue stained material was the appearance of a number of dense spherical bodies in the cytoplasm of the zygote (Fig 2). In organisms in which nuclear divisions had occurred the nuclei were generally well separated and mainly found close to the periphery of the organism (Fig. 3). At this stage of development the spatial relationship between the intracellular inclusions had changed so that now the dense bodies and the polysaccharide granules were evenly distributed thoughout the cytoplasm (Fig 4).

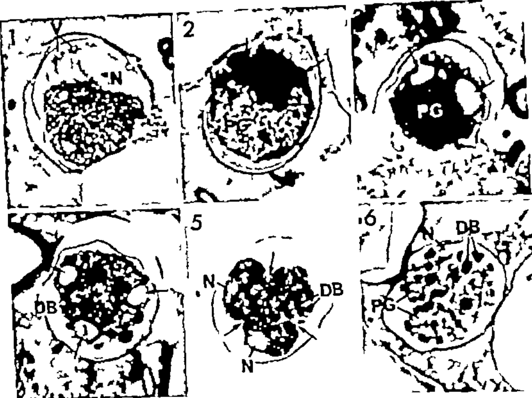
In sections prepared from material obtained after 24 hours of sporulation it was possible to find a number of organisms in which the zygote had divided and formed four sporoblasts (Fig 6) It would appear that the four sporoblasts were formed simultaneously (Fig 5).

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In thin sections the zygote of unsporulated oocysts (0 hour material) was found to be limited by a single unit membrane (Fig 8). The outline of the organism was irregular with a number of invaginations of the limiting membrane. Typical inactive micropores (13) were present on this membrane The cytoplasm of the zygote contained a number of polysaccharide granules which, as previously observed by light microscopy were densely packed into one portion of the cytoplasmic mass (Fig. 7) A number of electron translucent vacuoles were present in association with the polysaccharide granules. The translucent vacuoles were also observed close to the periphery of the organism together with a number of mitochondria. Some rough endoplasmic reticulum and a few Golgi bodies were observed in the portion of cytoplasm which was free from polysaccharide granules (Fig 7). The nucleus was present in the same region where it appeared as a large diffuse structure, with an indistinct limiting membrane and lacking dense patches of chromatin (Figs 7 and 9) In a few cases, a nucleolus was observed.

The ultrastructural changes occurring within the zygote prior to its division into sporoblasts were examined in material which had sporulated for 12 and 24 hours. The shape of the cytoplasmic mass of the zygote was found to remain unchanged (Fig 13), although certain organisms did show an increase in the number and extent of the invaginations of their limiting membranes (Fig 14). It appeared that these invaginations could form



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In the cytoplasm the amount of rough endoplasmic reticulum and the number and apparent activity of the Golgi bodies were increased (Figs 13 17 and 19). The number of free ribosomes also increased and polyribosomes were noticed. A number of

amorphous spherical bodies (dense bodies) were now present in the cytoplasm (Fig 13). These bodies apparently consisted of randomly oriented, densely packed membranes (Fig 18) and they could develop into large structures up to 3 μm in diameter (Fig 13). A Golgi body was often present in the vicinity of the developing dense bodies. At this stage, the large, diffuse nucleus appeared to have divided into two smaller nuclei with more distinct nuclear membranes (Fig. 13). Centriole-like structures and intra-nuclear poles were occasionally observed in association with the nuclei at this stage (Figs 10 11 and 12). After nuclear division, the nuclei apparently separated and they were now present at opposite sides of the cytoplasmic mass (Fig 17). Also at this time, the cytoplasmic organelles appeared to have been redistributed with the polysaccharide granules and dense bodies now present throughout the cytoplasm of the organism (Fig 17).

After this stage in the development, the cytoplasmic mass divided and gave rise to the four sporoblasts. This appeared to be accomplished by invaginations of the limiting membrane which isolated four individual areas of cytoplasm (Figs 20 and 21). In the majority of cases the four sporoblasts seemed to be formed simultaneously (Fig 20).

Fig 7 A section through an unsporulated oocyst (0 hours). A number of polysaccharide granules, electron translucent vacuoles, Golgi bodies, a few strands of rough endoplasmic reticulum, and a nucleus are present within the cytoplasmic mass. A nucleolus can be present although not shown in this section. 7500 ×

Fig 8 An enlargement of part of the periphery of a zygote showing it to be limited by a single unit membrane. 90 000 ×

Fig 9 An enlargement of part of the periphery of the nucleus seen in Fig. 7. Note the indistinct nuclear membranes. 90 000 ×

Fig 10 A detail from a zygote in which a centriole-like structure with nine microtubules (arrows) arranged in a circle round a central microtubule is seen. 90 000 ×

Fig 11 An enlargement of part of the nucleus shown in Fig. 12. Two longitudinally sectioned centriole-like structures and microtubules (arrows) radiating from the nuclear pole can be seen. 90 000 ×

Fig 12 A section through an organism with a dividing nucleus. Part of the intranuclear spindle (arrow) is illustrated. 30 000 ×

Fig 13 A section through an oocyst after 12 hours sporulation. In addition to the organelles present at the earlier stage the cytoplasm now contains a number of dense bodies. 7500 ×

Fig 14 Part of the periphery of a zygote. Bodies with an amorphous content seem to bud off and phagocytic vacuoles appear to be formed at the surface of this organism. 15 000 ×

Fig 15 A detail from the periphery of a zygote. An invagination of the limiting membrane is shown which may represent a stage in the formation of a phagocytic vacuole. 90 000 ×

Fig 16 In this micrograph an electron translucent vacuole is seen to protrude from the surface of the zygote. 45 000 ×

Fig 17 A section through an organism in which two nuclei are present. The nuclei are situated at opposite sides of the cytoplasmic mass. 7500 ×

Fig 18 A section through the periphery of a dense body showing it to consist of densely packed unit membranes. 90 000 ×

Fig 19 A detailed view of an active Golgi body present in an organism similar to that in Fig 13. 45 000 ×

Fig 20 A section of an organism in which the limiting membrane is seen to show invaginations at certain points (arrows) thus forming the individual sporoblasts. 10 000 ×

Fig 21 An enlargement of part of Fig 20. A deep invagination of the limiting membrane is illustrated. 30 000 ×

Fig 22 Part of a section in which three of the four newly formed sporoblasts are included. Each sporoblast contains a nucleus, a number of dense bodies, polysaccharide granules, electron translucent vacuoles, Golgi bodies, and a few strands of rough endoplasmic reticulum. 15 000 ×

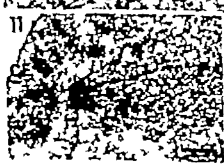
Fig 23 A section through sporoblasts at a slightly later developmental stage than those in Fig 22. In these sporoblasts the cytoplasm is more condensed although the organelle complement is unchanged. 15 000 ×

Fig 24 This micrograph shows part of a sporoblast in which the nucleus with associated Golgi bodies and strands of rough endoplasmic reticulum can be seen. Note the polysaccharide granules lined up around the dense body. 30 000 ×

Fig 25 Part of the periphery of a sporoblast in which a number of unit membranes with associated strands of rough endoplasmic reticulum are seen. 90,000 ×

Fig 26 Part of a section through the periphery of a sporoblast at a slightly later stage of development than that in Fig 25. This organism is limited by two membranes and a micropore is seen associated with the inner limiting membrane. 90 000 ×


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LM


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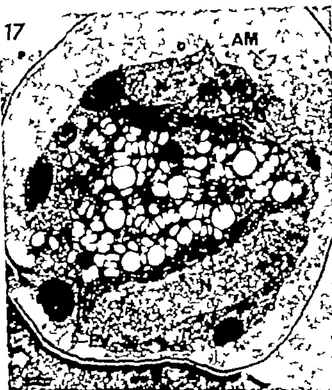
Fig 22 Part of a section in which three of the four newly formed sporoblasts are included Each sporoblast contains a nucleus, a number of dense bodies, polysaccharide granules, electron translucent vacuoles, Golgi bodies, and a few strands of rough endoplasmic reticulum. 15 000 ×

Fig 23 A section through sporoblasts at a slightly later developmental stage than those in Fig. 22. In these sporoblasts the cytoplasm is more condensed although the organelle complement is unchanged 15 000 ×

Fig 24 This micrograph shows part of a sporoblast in which the nucleus with associated Golgi bodies and strands of rough endoplasmic reticulum can be seen Note the polysaccharide granules lined up around the dense body 30 000 ×

Fig 25 Part of the periphery of a sporoblast in which a number of unit membranes with associated strands of rough endoplasmic reticulum are seen 90 000 ×

Fig 26 Part of a section through the periphery of a sporoblast at a slightly later stage of development than that in Fig. 25 This organism is limited by two membranes and a micropore is seen associated with the inner limiting membrane. 90 000 ×

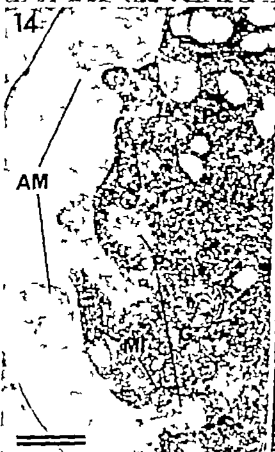

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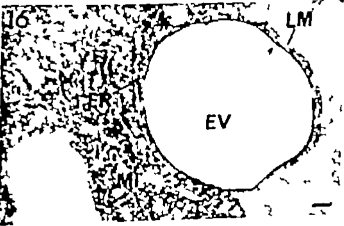

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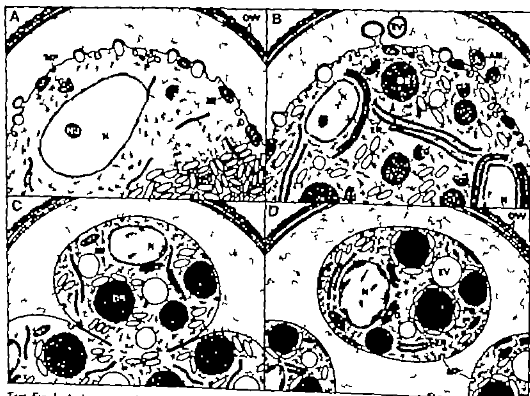
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The sporoblasts appeared as spherical structures limited by a unit membrane. They contained a nucleus, a number of randomly distributed polysaccharide granules, Golgi bodies, mitochondria, dense bodies and strands of rough endoplasmic reticulum (Figs 22 and 24). In certain cases, a number of polysaccharide granules were lined up around the periphery of the dense bodies (Fig 24). As the sporoblast developed, a number of strands of the rough endoplasmic reticulum were observed below the limiting membrane (Fig. 25), and the organism had become enveloped by a second unit membrane (Fig. 26). The inner limiting membrane could be identified as the plasmalemma because of associated micropores (Fig. 26). The cytoplasm of the sporoblast had become more condensed although the organelle complement was similar to that of the earlier stage (cf Figs 22 and 23). A diagrammatical representation of the changes observed during the initial development of the oocysts is given in Text Fig 1



Text Fig 1 A diagrammatical representation of the changes observed during the development of the zygote and formation of the sporoblasts A an unsporulated oocyst (0 hours), B a sporulating oocyst (12 hours), C the formation of the sporoblasts, D early sporoblasts

DISCUSSION

The oocysts were maintained at 4 C during concentration and storage, this prevents sporulation although they remain viable for some time. This non-sporulation but retention of viability of oocysts has also been reported for *E. magna* (20)

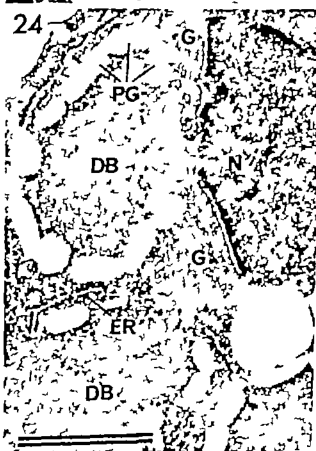
The only difference we could note between the forming oocysts of *E. brunetti* in the intestinal tissue (11) and that observed in the sample processed directly from 4° C was a contraction of the zygote away from the oocysts wall and a congregation of the polysaccharide granules in one portion of the organism. The structure of the oocysts containing the zygote is similar to that described for *E. necatrix* (7) except that the oocysts of *E. necatrix* showed no congregation of their polysaccharide granules. The presence of a single unit membrane limiting the zygote is similar to that reported for *E. necatrix* (7) and *E. tenella* (5) but differs from that reported for *E. alexchdza* by *Dubremetz et al.* (6). *Dubremetz et al.* however used the freeze-etching technique for



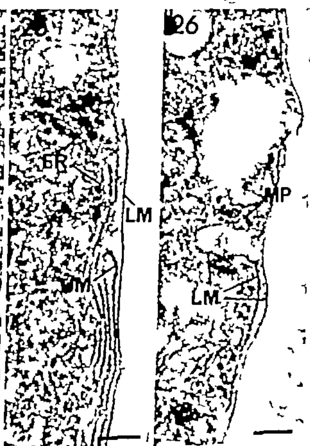
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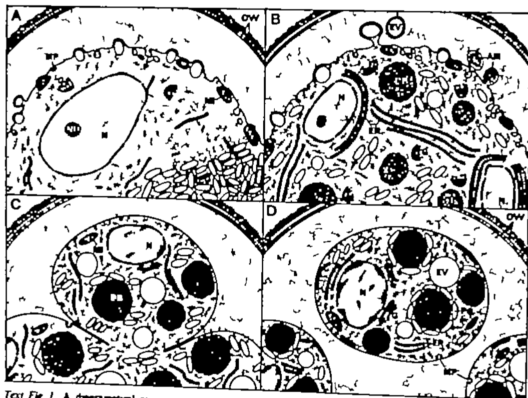


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Text Fig. 1. A diagrammatical representation of the changes observed during the development of the zygote and formation of the sporoblasts. A. an unsporulated oocyst (0 hours). B. a sporulating oocyst (12 hours), C. the formation of the sporoblasts, D. early sporoblasts.

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In the zygote we noted an aggregation of the polysaccharide granules within a certain region of the organism It is possible that this aggregation could be a preparative artifact (e g due to centrifugation) but this seems unlikely since the granules become redispersed as sporulation proceeds further

The initiation of sporulation appears to be accompanied by an increase in the metabolic activity and protein synthesis as evidenced by the increase in the amount of rough endoplasmic reticulum the number of Golgi bodies, and the appearance of polyribosomes. This apparent high metabolic activity of oocysts in the early sporulation phase has also been reported for certain other members of the Sporozoa (3 25). This early development is also accompanied by the appearance of the dense bodies which represent the anlagen of the refractile bodies of the sporozoites (14). Certain of the structures marked »mitochondrion?« in the freeze etching study of *E nieschulzi* (6) may represent these bodies which could be difficult to identify in replicas of the freeze etched material The refractile bodies are reported to contain protein or lipoprotein moieties (16 24). We have observed accumulations of unit membranes associated with the anlagen of the refractile bodies and this is certainly consistent with the lipoprotein nature of these organelles. The refractile bodies of *Eimeria* spp. are believed to have a function similar to the crystalloid bodies observed in a number of other members of the Sporozoa (19) These crystalloid bodies have been shown to contain both lipids and proteins (4 23) and they thus resemble refractile bodies in chemical composition.

Some vacuoles were observed close to the limiting membrane of the zygote. Although we cannot prove that they are true phagocytic vacuoles, they could represent an uptake of material from the surrounding medium We also noticed some electron translucent vacuoles which were apparently budding off from the surface of the zygote. These vacuoles are similar in appearance to those observed in developing macrogametes (10) and as proposed in that study their function may be one of waste disposal Vacuoles have also been observed budding off in the oocysts of *Leucocytozoon dubreuili* and in this case a waste disposal function was also proposed (26). It is possible that the amorphous structures observed budding off from the periphery have a similar function to that of the electron translucent vacuoles.

The presence of micropores on the zygotes and sporoblasts has been discussed previously (13).

In the oocysts of *E brunetti* the large diploid nucleus of the zygote possesses an indistinct nuclear membrane. This observation has also been reported for the nucleus within the oocysts of *Plasmodium berghei yoelii* (15). In our study the reduction division could not be definitely recognized, but the haploid nuclei of the later stages were found to be smaller and with very distinct nuclear membranes. Centrioles and intra nuclear spindle poles with radiating microtubules were involved in nuclear division in the zygote of *E brunetti* as they were for the endogenous forms previously reported (8 9). In the case of the oocysts of other members of the Sporozoa a similar involvement of these organelles has also been reported (15 21 25). In the oocysts of *E brunetti* the nuclear membrane appears to remain intact during nuclear division. This differs from the light microscope observations reported for *E tenella* which indicated that the nuclear membrane disappeared and that chromosomes could be seen (2) In the sporulating oocysts of *E brunetti* it would appear that the nucleus divides twice and gives rise to four nuclei. These nuclei are situated at the periphery of the zygote and are well separated from each other This situation is similar to that reported for the sporulating oocysts of *E debliecki* (24)

In *E brunetti*, the formation of the four sporoblasts occurs simultaneously by division of the cytoplasmic mass. This process is similar to that reported for *E tenella* (2) and *E debliecki* (24) The sporoblasts of *E brunetti* were formed by invaginations of the limiting membrane of the organism. This observation is similar to that reported for *Hepatozoon domerguei* (25) but it differs from that reported for other members of the Sporozoa in which sporoblast formation was found to occur by a coalescence of aligned vesicles within the cytoplasmic mass (17 18 21 22). From our study it appears that the formation of the uninucleate sporoblasts is similar to that reported for *E debliecki* (24) and that the final nuclear division occurs within the developing sporoblast.

We are indebted to the *Central Veterinary Laboratory* Ministry of Agriculture, Fisheries and Food, New Haw Weybridge, Surrey England, for supplying a pure sample of oocysts of *E brunetti* and to *K L Fennestad* V M D Statens Seruminstitut, for provision and maintenance of the chickens We gratefully acknowledge Dr *J Blom* for assistance with the light microscopy Mrs *H Ravn* and Mrs *J Berg* for technical assistance, and Miss *A G Overgaard* and Mr *F Laursen* for photographic assistance

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The work was supported by grants from the *World*

Acta path microbiol scand Sect B, 86 13–24 1978

LIGHT AND ELECTRON MICROSCOPY ON THE SPORULATION OF THE OOCYSTS OF *EIMERIA BRUNETTI*

II Development Into the Sporocyst and Formation of the Sporozoite

D J P FERGUSON[1,2] A BIRCH ANDERSEN[2] W M HUTCHISON[3] and J CHR SIIM[1]

FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut, Copenhagen, Denmark, and Department of Biology[3] University of Strathclyde, Glasgow Scotland

Ferguson, D J P Birch-Andersen, A Hutchison, W M & Siim, J Chr Light and electron microscopy on the sporulation of the oocysts of *Eimeria brunetti* II Development into the sporocyst and formation of the sporozoite Acta path microbiol scand Sect B, 86 13–24 1978

The later stages of sporulation in oocysts of *Eimeria brunetti* were examined in samples which had been allowed to sporulate at 27° C for 24 36 and 48 hours It was observed that the sporoblasts became ellipsoidal and the nucleus underwent the final division A nucleus with associated Golgi bodies was now observed at either end of the organism The cytoplasm was limited by two unit membranes and contained rough endoplasmic reticulum, dense bodies, electron translucent vacuoles and mitochondria The first evidence of sporozoite formation was the appearance of a dense plaque at either end of the organism This appeared in the vicinity of the nuclei, and adjacent to the limiting membrane of the sporoblast At this stage the sporocyst wall was still unformed Then the two sporozoites were formed from opposite ends of the organism by growth of the dense plaques and invaginations of the plasmalemma which thus formed the pellicles of the developing sporozoites A conoid and subpellicular microtubules were observed at this stage and as development continued, a number of vacuoles were found between the nucleus and the conoid These vacuoles constituted the precursors of the rhoptries and micronemes At the same stage a large dense body had appeared within the forming sporozoite As the sporozoite developed, this body the anterior *refractile body* is followed by the nucleus and another dense body which formed the posterior refractile body During this period the thin sporocyst wall was formed and Stieda and sub-Stieda bodies were now present at one end of the sporocyst Each mature sporocyst contained two sporozoites

Key words *Eimeria brunetti* sporulation, sporozoite formation, sporocyst, ultrastructure,

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To the best of our knowledge, no detailed ultrastructural description of the sporulation of any member of the Eimeriidae has ever been reported. Previously we have described the initial stages (i e development of the zygote and sporoblast formation) of the process within the oocysts of *Eimeria brunetti* (9). In this report the final stages in sporulation (i e development of the sporocyst and formation of the sporozoites) will be described and the ultrastructural changes observed during this part of the sporulation of *E brunetti* will be compared with those reported to occur for other members of the Sporozoa.

Work initiated while a Wellcome Trust Travelling Research Fellow and completed as a Danish Medical Research Council Fellow

sporocysts and during this time many also showed that sporozoites had formed within the sporocysts. After 48 hours of sporulation, the predominant feature was sporocysts, which contained two fully formed sporozoites, although a few organisms were still seen to be at the zygote, sporoblast, or early sporocyst stages of development.

In sections stained with toluidine blue it was seen that as the sporoblast developed it became more ellipsoidal (cf. Figs 1 and 2). A nucleus was present at either end of the organism, the cytoplasm of which contained numerous dense bodies and polysaccharide granules (Fig. 2).

As the sporoblast further developed into the sporocyst a thin sporocyst wall was formed and in this a prominent Stieda body was observed at one end of the sporocyst (Fig. 3).

Details of sporozoite formation from the sporoblast stage cannot be observed with the light microscope, although it was possible to observe sporozoites being formed at opposite ends of the organism (Fig. 3). Two elongate sporozoites were found within each mature sporocyst. Each sporozoite possessed a centrally located nucleus and in the cytoplasm, anterior and posterior to this, two prominent dense bodies (refractile bodies) were present (Fig. 4).

Electron Microscopy

The structure of the sporoblast has been described previously (9). As the sporoblast further developed it became ellipsoidal and by then a nucleus with associated Golgi bodies was present at opposite ends of the organism (Fig. 5). The cytoplasm contained a number of dense bodies, polysaccharide granules, electron translucent vacuoles, mitochondria and strands of rough endoplasmic reticulum (Fig. 5). The organism was found to be enveloped by two membranes, the inner of which possessed micropores of the inactive type (Fig. 6) (8). The outer membrane had lost its unit membrane character (Fig. 6) and was associated with the formation of the sporocyst wall.

Sporozoite formation seemed to be initiated by the appearance of a dense plaque at either end of the organism in the vicinity of a nucleus and in association with the limiting membranes of the organism (Fig. 5). A conoid appeared to be formed in the central region of each plaque and some microtubules were present in the cytoplasm adjacent to them (Figs 7 and 8). Pieces of unit membranes could also be distinguished within the dense material of the plaque (Figs 7 and 8). A nuclear pole was observed at this stage. It was found to be directed towards the dense plaque and a centriole-like structure was occasionally seen close to it (Fig. 8).

The initiation of the Stieda body formation was observed on the outer limiting membrane at one end of the organism (Fig. 8). The Stieda body was always observed to be formed close to the site of formation of one of the sporozoites (Fig. 8). No

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Fig. 5. A longitudinal section through a sporoblast. The cytoplasm contains a nucleus with associated Golgi body at either end of the organism (details of the Golgi bodies have been confirmed at higher magnification). A number of polysaccharide granules, dense bodies, mitochondria, electron translucent vacuoles, and a few strands of rough endoplasmic reticulum are also present. Note the dense plaque present at either end of the organism (arrows). 15,000 ×

Fig. 6. A section through part of the periphery of an organism similar to that in Fig. 5. Note the two limiting membranes and the micropore associated with the inner of these. 90,000 ×

Fig. 7. Part of a section through a sporoblast showing the formation of a dense plaque. The plaque consists of two closely applied unit membranes (arrow) and has microtubules in close association. The inner limiting membrane (plasmalemma) and a very early stage of the Stieda body formation can also be seen. 90,000 ×

Fig. 8. A section through part of a sporoblast showing the initiation of sporozoite formation. A nuclear pole, with associated centriole-like structure, is directed towards a dense plaque (arrow). Note the conoid present close to the plaque. 90,000 ×

Fig. 9. A section through part of a sporoblast showing two Golgi bodies situated close to the nucleus. Note the invagination of the limiting membrane (arrow) which is associated with sporozoite formation. 30,000 ×

Fig. 10. A longitudinal section through a sporoblast showing a nucleus at either end of the organism. A conoid is present and an invagination of the limiting membrane which forms the anterior of a developing sporozoite can be seen (arrow). 7,500 ×

Fig. 11. A section through part of a sporoblast. The posterior growth of the plaque is shown (arrows) and a precursor of a rhoptry or micronome is also present. 30,000 ×

Fig. 12. This section shows the invagination of the limiting membrane of the sporoblast (arrow) and the growth of the plaque towards the nucleus. Vacuoles are present between the nucleus and the anterior of the plaque. 30,000 ×

Fig. 13. A cross section through a sporoblast showing the formation of a sporozoite. The invaginations of the limiting membrane and plaque can be seen (arrows). 30,000 ×

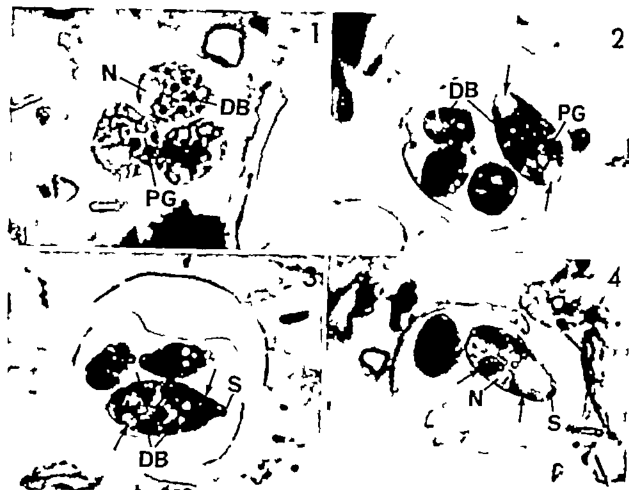
MATERIALS AND METHODS

The materials and methods were as described previously (3 9). In this study of the later stages of sporulation, oocysts which had been allowed to sporulate for 24 36 and 48 hours were examined The results published are based on the examination of more than 900 electron micrographs.

RESULTS

Light Microscopy

After 24 hours of sporulation, a number of oocysts contained sporoblasts which were developing into sporocysts. After 36 hours of sporulation, the majority of organisms had formed their



Figures 1–4 are light micrographs and Figures 5–25 are electron micrographs of sections and illustrate the development of the sporoblast into the sporocyst and the formation of the sporozoites within oocysts of *E brunetti*

A double bar (=) on a figure represents 1 µm and a single bar (–) 100 nm.

The following abbreviations are used throughout. C = conoid CE = centriole, CM = cytoplasmic mass, DB = dense body (precursor of the refractile body) ER = rough endoplasmic reticulum, EV = electron translucent vacuole; G = Golgi body LM = limiting membrane, MI = mitochondrion, MN = microneme; MT = microtubule, N = nucleus, NP = nuclear pole, OW = oocyst wall, PG = polysaccharide granule; PL = pellicle; PP = posterior pore, RB = refractile body RH = rhoptry RM = residual cytoplasmic mass, S = Stieda body; SP = sporozoite SS = sub-Stieda body SW = sporocyst wall V = vacuole (precursor of a rhoptry or microneme)

Fig 1 A section in which three of the four spherical sporoblasts are included Each sporoblast contains a nucleus, and a number of polysaccharide granules and dense bodies 2000 ×

Fig 2 A section which contains all four sporoblasts At this stage the sporoblasts are ellipsoidal and in the one cut longitudinally a nucleus is present at either end of the organism (arrows) 2000 ×

Fig 3 A section which contains a longitudinally cut sporoblast. The formation of a sporozoite at either end of the organism can be seen (arrows) Note the Stieda body present at one end of the sporoblast. 2000 ×

Fig 4 A section in which is included a longitudinal cut through a fully sporulated sporocyst The two sporozoites and the Stieda body are shown Note that the sporozoites have a refractile body both anterior and posterior to the nucleus (arrows). 2000 ×

sporocysts and during this time many also showed that sporozoites had formed within the sporocysts. After 48 hours of sporulation, the predominant feature was sporocysts, which contained two fully formed sporozoites, although a few organisms were still seen to be at the zygote, sporoblast, or early sporocyst stages of development.

In sections stained with toluidine blue it was seen that as the sporoblast developed it became more ellipsoidal (cf Figs 1 and 2). A nucleus was present at either end of the organism, the cytoplasm of which contained numerous dense bodies and polysaccharide granules (Fig. 2).

As the sporoblast further developed into the sporocyst a thin sporocyst wall was formed and in this a prominent Stieda body was observed at one end of the sporocyst (Fig. 3)

Details of sporozoite formation from the sporoblast stage cannot be observed with the light microscope, although it was possible to observe sporozoites being formed at opposite ends of the organism (Fig. 3). Two elongate sporozoites were found within each mature sporocyst. Each sporozoite possessed a centrally located nucleus and in the cytoplasm, anterior and posterior to this, two prominent dense bodies (refractile bodies) were present (Fig. 4)

Electron Microscopy

The structure of the sporoblast has been described previously (9). As the sporoblast further developed it became ellipsoidal and by then a nucleus with associated Golgi bodies was present at opposite ends of the organism (Fig 5). The cytoplasm contained a number of dense bodies, polysaccharide granules, electron translucent vacuoles, mitochondria and strands of rough endoplasmic reticulum (Fig 5). The organism was found to be enveloped by two membranes, the inner of which possessed micropores of the inactive type (Fig 6) (8). The outer membrane had lost its unit membrane character (Fig 6) and was associated with the formation of the sporocyst wall

Sporozoite formation seemed to be initiated by the appearance of a dense plaque at either end of the organism in the vicinity of a nucleus and in association with the limiting membranes of the organism (Fig 5). A conoid appeared to be formed in the central region of each plaque and some microtubules were present in the cytoplasm adjacent to them (Figs 7 and 8). Pieces of unit membranes could also be distinguished within the dense material of the plaque (Figs 7 and 8). A nuclear pole was observed at this stage. It was found to be directed towards the dense plaque and a centriole-like structure was occasionally seen close to it (Fig. 8).

The initiation of the Stieda body formation was observed on the outer limiting membrane at one end of the organism (Fig. 8). The Stieda body was always observed to be formed close to the site of formation of one of the sporozoites (Fig. 8). No

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Fig 5 A longitudinal section through a sporoblast. The cytoplasm contains a nucleus with associated Golgi body at either end of the organism (details of the Golgi bodies have been confirmed at higher magnification). A number of polysaccharide granules, dense bodies, mitochondria, electron translucent vacuoles, and a few strands of rough endoplasmic reticulum are also present. Note the dense plaque present at either end of the organism (arrows). 15,000 ×

Fig 6 A section through part of the periphery of an organism similar to that in Fig. 5. Note the two limiting membranes and the micropore associated with the inner of these. 90,000 ×

Fig 7 Part of a section through a sporoblast showing the formation of a dense plaque. The plaque consists of two closely applied unit membranes (arrow) and has microtubules in close association. The inner limiting membrane (plasmalemma) and a very early stage of the Stieda body formation can also be seen. 90,000 ×

Fig 8 A section through part of a sporoblast showing the initiation of sporozoite formation. A nuclear pole, with associated centriole-like structure, is directed towards a dense plaque (arrow). Note the conoid present close to the plaque. 90 000 ×

Fig 9 A section through part of a sporoblast showing two Golgi bodies situated close to the nucleus. Note the invagination of the limiting membrane (arrow) which is associated with sporozoite formation. 30,000 ×

Fig 10 A longitudinal section through a sporoblast showing a nucleus at either end of the organism. A conoid is present and an invagination of the limiting membrane which forms the anterior of a developing sporozoite can be seen (arrow). 7,500 ×

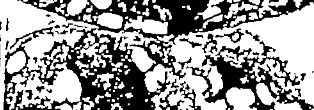
Fig 11 A section through part of a sporoblast. The posterior growth of the plaque is shown (arrows) and a precursor of a rhoptry or microneme is also present. 30,000 ×

Fig 12 This section shows the invagination of the limiting membrane of the sporoblast (arrow) and the growth of the plaque towards the nucleus. Vacuoles are present between the nucleus and the anterior of the plaque. 30,000 ×

Fig 13 A cross section through a sporoblast showing the formation of a sporozoite. The invaginations of the limiting membrane and plaque can be seen (arrows). 30,000 ×



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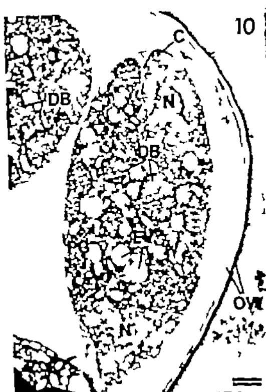
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specific structures appeared to be involved in either Stieda body or sporocyst wall formation. The material for these structures appeared to be coalesced onto the outer limiting membrane of the organism. At the initiation of sporozoite formation the sporocyst wall was still unformed.

As sporozoite formation continued, the plaque developed posteriorly into the cytoplasmic mass (Figs. 9, 11 and 12). The two sporozoites were formed from opposite ends of the parent body (Figs. 5, 10 and 16). At this stage a number of Golgi bodies with associated vesicles were observed around each nucleus (Fig. 9). The pellicle of the developing sporozoite consisted of the invaginated limiting membrane of the cytoplasmic mass and adjacent to this membrane was the plaque which appeared as two closely applied unit membranes (Figs. 12, 13 and 15). In cross section through the early stages, the plaques appeared as a number of distinct patches (Fig. 17). Microtubules were observed in the cytoplasm in close association with the pellicle (Fig. 17). A number of unit membrane enclosed vacuoles, varying in size and with granular contents, were observed between the nucleus a[nd] the anterior part of the developing sporozoite (Fi[g.] 14). They probably represent the precursors of t[he] rhoptries and micronemes. In the later stages [of] sporozoite development, ducts were observed [in] continuity with some of the larger vacuoles. It w[as] also possible at this stage to observe inactiv[e] micropores on the limiting membrane of t[he] cytoplasmic mass of the organism (Fig. 13).

As the sporozoites grew posteriorly a large den[se] body was seen to be included in the cytoplasm. Th[is] body was found anterior to the nucleus and cou[ld] now be termed the anterior refractile body (Fig. 16). In the later stages of sporozoite formation a larg[e] posterior refractile body was also observed (Fig. 19). Both of these refractile bodies seemed to be forme[d] by a coalescence of the smaller dense bodies, whic[h] at earlier stages were distributed in the cytoplasm (Fig. 5). In addition, mitochondria, rhoptries, mi[-]cronemes, rough endoplasmic reticulum and a fe[w] polysaccharide granules were observed in the cyto[-]plasm of the developing sporozoites (Figs. 19, 2[0] and 21). These sporozoites seemed to remai[n]

Fig. 14. A section through part of a sporoblast. The Stieda body and the anterior part of a forming sporozoite can be seen. Note the vacuoles present between the nucleus and conoid, and the pellicle which is formed by the dense plaque and the invaginated limiting membrane of the sporoblast. 45 000 ×

Fig. 15. A detail showing the invagination of the limiting membrane of the sporoblast. The pellicle of the developing sporozoite is formed by this membrane in association with the dense plaque (arrows). Note also the presence of a single limiting membrane on the cytoplasmic mass of the sporoblast. 90 000 ×

Fig. 16. A longitudinal section through an early sporocyst. The two partially formed sporozoites can be seen. The extent of the invagination of the limiting membrane of the organism and the growth of the plaque is marked (arrows). It is illustrated how the anterior refractile body and the nucleus of each sporozoite is enclosed at this stage. 15 000 ×

Fig. 17. A cross section through part of the periphery of an early sporozoite. The organism is enclosed by a pellicle consisting of a limiting unit membrane and the plaque which, at this stage, appears as a number of distinct patches (arrows). Note the microtubules associated with the patches. 90 000 ×

Fig. 18. A section through part of a late sporocyst. A sporozoite is shown to be attached to the residual cytoplasmic mass at the posterior end. Note that the inner layer of the pellicle of the sporozoite forms the posterior pore (arrows). 90 000 ×

Fig. 19. A longitudinal section through a sporozoite. Th[e] cytoplasm contains a conoid and a number of rhoptrie[s,] micronemes and polysaccharide granules. Two refracti[le] bodies are also present, situated on either side of th[e] nucleus. 15 000 ×

Fig. 20. A cross section through the anterior part of [a] sporozoite. The rhoptries and micronemes can be see[n] enclosed by a pellicle. The sporocyst wall is also seen. 30 000 ×

Fig. 21. A cross section through a sporozoite at the lev[el] of the nucleus. The nucleus and Golgi bodies can be see[n] enclosed by a pellicle. The sporocyst wall is also present. 30 000 ×

Fig. 22. A cross section through a fully forme[d] sporocyst. The two cross cut sporozoites and the residua[l] cytoplasmic mass can be seen. 15 000 ×

Fig. 23. A longitudinal section through the anterior par[t] of a mature sporocyst. The Stieda body is situated in th[e] aperture of the sporocyst wall. Note the presence of th[e] sub-Stieda body. 30 000 ×

Fig. 24. A section through part of the periphery of a sporocyst showing the pellicle of a sporozoite and th[e] structure of the sporocyst wall. 90 000 ×

Fig. 25. A section through part of the periphery of one of the sporozoites in the sporocyst shown in Fig. 22. Note that the sporozoite is limited by a pellicle and the residual cytoplasmic mass by a single unit membrane. 90 000 ×


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specific structures appeared to be involved in either Stieda body or sporocyst wall formation The material for these structures appeared to be coalesced onto the outer limiting membrane of the organism At the initiation of sporozoite formation the sporocyst wall was still unformed.

As sporozoite formation continued the plaque developed posteriorly into the cytoplasmic mass (Figs 9 11 and 12) The two sporozoites were formed from opposite ends of the parent body (Figs. 5 10 and 16) At this stage a number of Golgi bodies with associated vesicles were observed around each nucleus (Fig 9) The pellicle of the developing sporozoite consisted of the invaginated limiting membrane of the cytoplasmic mass and adjacent to this membrane was the plaque which appeared as two closely applied unit membranes (Figs 12 13 and 15) In cross section through the early stages, the plaques appeared as a number of distinct patches (Fig 17) Microtubules were observed in the cytoplasm in close association with the pellicle (Fig 17) A number of unit membrane enclosed vacuoles, varying in size and with granular contents, were observed between the nucleus a[illegible] the anterior part of the developing sporozoite (Fi[illegible] 14). They probably represent the precursors of t[illegible] rhoptries and micronemes. In the later stages [illegible] sporozoite development, ducts were observed [illegible] continuity with some of the larger vacuoles. It w[illegible] also possible at this stage to observe micr[illegible] micropores on the limiting membrane of t[illegible] cytoplasmic mass of the organism (Fig 13).

As the sporozoites grew posteriorly a large den[illegible] body was seen to be included in the cytoplasm. Th[illegible] body was found anterior to the nucleus and cou[illegible] now be termed the anterior refractile body (Fig 1[illegible] In the later stages of sporozoite formation a larg[illegible] posterior refractile body was also observed (Fig. 1[illegible] Both of these refractile bodies seemed to be form[illegible] by a coalescence of the smaller dense bodies, whi[illegible] at earlier stages were distributed in the cytopla[illegible] (Fig. 5). In addition, mitochondria, rhoptries, m[illegible] cronemes rough endoplasmic reticulum and a fe[illegible] polysaccharide granules were observed in the cyt[illegible] plasm of the developing sporozoites (Figs. 19 [illegible] and 21) These sporozoites seemed to remai[illegible]

Fig 14 A section through part of a sporoblast. The Stieda body and the anterior part of a forming sporozoite can be seen. Note the vacuoles present between the nucleus and conoid, and the pellicle which is formed by the dense plaque and the invaginated limiting membrane of the sporoblast 45 000 ×

Fig 15 A detail showing the invagination of the limiting membrane of the sporoblast. The pellicle of the developing sporozoite is formed by this membrane in association with the dense plaque (arrows) Note also the presence of a single limiting membrane on the cytoplasmic mass of the sporoblast. 90 000 ×

Fig 16 A longitudinal section through an early sporocyst. The two partially formed sporozoites can be seen The extent of the invagination of the limiting membrane of the organism and the growth of the plaque is marked (arrows) It is illustrated how the anterior refractile body and the nucleus of each sporozoite is enclosed at this stage 15 000 ×

Fig 17 A cross section through part of the periphery of an early sporozoite. The organism is enclosed by a pellicle consisting of a limiting unit membrane and the plaque which at this stage appears as a number of distinct patches (arrows) Note the microtubules associated with the patches 90 000 ×

Fig 18 A section through part of a late sporocyst A sporozoite is shown to be attached to the residual cytoplasmic mass at the posterior end Note that the inner layer of the pellicle of the sporozoite forms the posterior pore (arrows) 90 000 ×

Fig 19 A longitudinal section through a sporozoite. T[illegible] cytoplasm contains a conoid and a number of rhoptr[illegible] micronemes and polysaccharide granules Two refract[illegible] bodies are also present, situated on either side of t[illegible] nucleus. 15 000 ×

Fig 20 A cross section through the anterior part of [illegible] sporozoite. The rhoptries and micronemes can be se[illegible] enclosed by a pellicle. The sporocyst wall is also see[illegible] 30 000 ×

Fig 21 A cross section through a sporozoite at the lev[illegible] of the nucleus. The nucleus and Golgi bodies can be see[illegible] enclosed by a pellicle The sporocyst wall is also presen[illegible] 30 000 ×

Fig 22 A cross section through a fully forme[illegible] sporocyst The two cross cut sporozoites and the residu[illegible] cytoplasmic mass can be seen. 15 000 ×

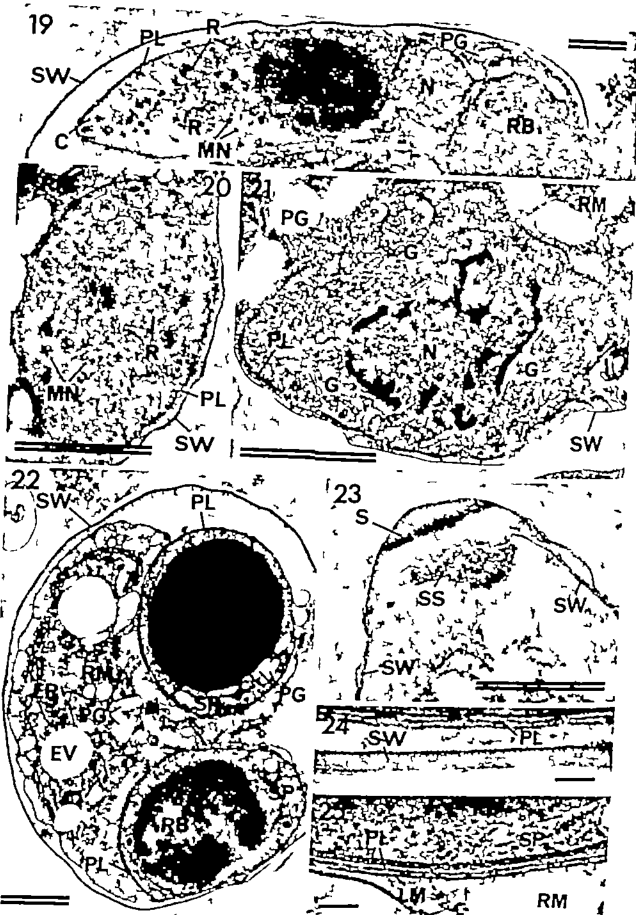
Fig 23 A longitudinal section through the anterior pa[illegible] of a mature sporocyst. The Stieda body is situated in th[illegible] aperture of the sporocyst wall Note the presence of th[illegible] sub-Stieda body 30 000 ×

Fig 24 A section through part of the periphery of [illegible] sporocyst showing the pellicle of a sporozoite and th[illegible] structure of the sporocyst wall 90 000 ×

Fig 25 A section through part of the periphery of one o[illegible] the sporozoites in the sporocyst shown in Fig 2[illegible] Note that the sporozoite is limited by a pellicle and the residual cytoplasmic mass by a single unit membrane 90 000 ×


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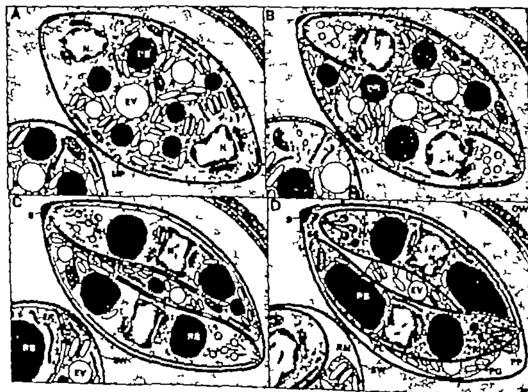


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attached to the residual mass for some time and during this period a posterior polar ring appeared to be formed by the inner layer of the pellicle (Fig. 18). The pellicle consisted of a single plus two closely applied unit membranes (Fig. 25).

After separation from the residual mass the two sporozoites were situated within the sporocyst orientated in opposite directions to each other. Generally a micropore of the inactive type was observed on each of the sporozoites. The residual mass from which the sporozoites had developed was limited by a unit membrane (Fig. 25) on which an inactive micropore could be occasionally observed. The residual mass as such consisted of a number of polysaccharide granules and electron translucent vacuoles, a few mitochondria and strands of rough endoplasmic reticulum (Fig. 22). During this development of the sporozoites the sporocyst wall had formed simultaneously (Fig. 22). A diagrammatical representation of the changes observed during this development is given in Text Fig. 1.

The mature sporocyst containing the sporozoites was limited by a wall (70 nm thick) (Fig. 24) which in certain organisms presented a laminated structure (Figs. 23 and 24). The sporocyst wall contained a round opening (720 nm diameter) at one end. The Stieda body was situated at this part of the wall where it appeared to form a plug- and cap-like structure sealing the opening (Fig. 23). The sub-Stieda body was present within the sporocyst immediately below the Stieda body (Fig. 23). It presented a granular appearance compared to the more amorphous character of the material of the latter (Fig. 23).



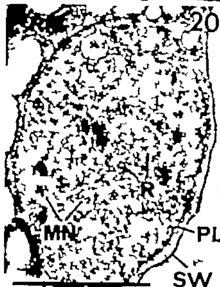
Text Fig. 1. A diagrammatical representation of the changes observed during the development of the sporoblast into the sporocyst with the simultaneous formation of the two sporozoites (A–C). The structure of the fully sporulated sporocyst is shown in D.

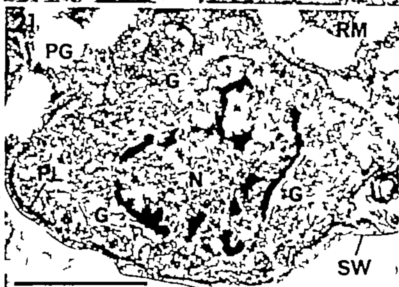
DISCUSSION

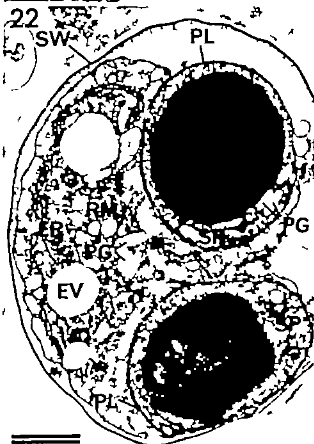
In *Eimeria brunetti* the late sporoblast was found to develop into an ellipsoidal-shaped structure. This is similar to that observed for the sporoblasts of *E. debliecki* (29) and *Coelotropha durchoni* (13). In addition, it was observed that the final nuclear division occurs at this stage, which also is similar to that reported for *E. debliecki* (29).

Sporozoite formation in *E. brunetti* was initiated by the formation of a dense plaque close to the limiting membrane in the vicinity of a nucleus.

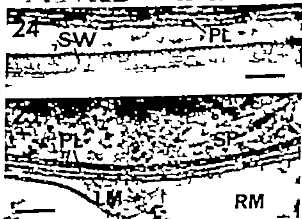

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sample of oocysts of *E. brunetti*, and to *K. L. Fennestad*, V.M.D. Statens Seruminstitut, for provision and maintenance of the chickens. We gratefully acknowledge Dr *J. Blom* for assistance with the light microscopy, Mrs *H. Rex* and Mrs *J. Berg* for technical assistance, and Mrs *A. G. Overgaard* and Mr *F. Lauesen* for photographic assistance.

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This is analogous to the initiation of sporozoite formation in other members of the Sporozoa (4 5 13 14 15 17 18 24 25 26 27 28 31). In the sporoblasts of the present material the nuclear pole was found to be retained and oriented towards the developing daughters. A similar situation existed in the schizont of *E brunetti* (6) The initiation of sporozoite formation was found to occur prior to the formation of the sporocyst wall. This condition differs from what has been reported for related families and genera, which also produce sporocysts but for which a fully formed sporocyst wall has been found to precede the formation of the sporozoite (2, 10 11 13 15 16 18) Whether or not this particular feature of sporozoite formation in *E brunetti* is typical of the genus *Eimeria* will have to await the examination of other members of this genus

The basic process of sporozoite formation as observed in *E brunetti* is similar to that described for other members of the Sporozoa. It differs, however from that reported for *Plasmodium* spp *Leucocytozoon* spp and *Haemoproteus metchnikovi* (4 5 24 25 26 28 31) The main difference between the two types of sporozoite formation is that the developing sporozoites of *E brunetti* are not formed from protrusions of the surface of the cytoplasmic mass but from invaginations of the limiting membranes. In the case of *E brunetti* a limited space is available to the developing sporozoites due to the simultaneous formation of the sporocyst wall, and this could probably explain why the sporozoite formation takes place by an invagination process. An inward growth rather than a budding off process for the developing sporozoites has also been reported for the sporogony of *C durchoni Diplauxis schrevelI D hatti* and *A eberthi* in which sporozoite formation also occurs within the confines of a sporocyst (13 15 17 18).

The asexual multiplication (schizogony) occurring in the endogenous forms of the Sporozoa has been reviewed by *Porchet Hennerė* (14) and *Aikawa & Sterling* (1) The present available knowledge indicates that schizogony can occur via two different types of basic processes. One in which daughter formation occurs at the mother cell s surface and the other where daughter formation occurs within the mother cell (endodyogeny and endopolygeny). The terms exogenèse (exogenesis) and endogenèse (endogenesis) respectively have been proposed to differentiate between these processes (30) The majority of species so far examined were found to undergo exogenesis while endogenesis has only been observed in a few genera (14). The sporogonic process as observed for *E brunetti* is similar to the exogenesis described for the endogenous forms of the Sporozoa. Thus it would appear that the asexual multiplication undergone by *E brunetti* is very similar whether it occurs in the endogenous forms by schizogony (6) or within the oocyst by sporogony In our experience, at least, no fundamental differences were observed.

The ultrastructure of the sporozoite of *E brunetti* with its anterior and posterior refractile bodies is similar to that reported for the sporozoites of other *Eimeria* spp (19 21).

The formation of the sporocyst wall and the Stieda body did not appear to be associated with any particular cytoplasmic organelle, such as the wall-forming bodies which participate in the formation of the oocyst wall (7) This differs from that reported for *Sarcocystis tenella* (11) and *A eberthi* (10) where »osmiophilic bodies« and »granular bodies« respectively were thought to be involved in sporocyst wall formation. For *E brunetti* the sporocyst wall and the Stieda body appeared to be formed by a gradual coalescence of material onto the outer limiting membrane of the developing sporocyst. The only opening present in the sporocyst wall is occupied by the Stieda body which forms a plug and cap-like structure over the gap The structure of the Stieda body resembles that reported for this organelle in *E larimerensis* (20) and the presence of a sub-Stieda body is also similar to what has been described for sporocysts of *E. larimerensis* and *E callospermophili* (20). The sporocyst wall of *E brunetti* is thin and composed of a single layer and this is similar to the structure of the sporocyst wall of *E larimerensis* (20). The structure of the sporocyst walls of the *Eimeria* spp. examined to date differs from that reported for the sporocysts of certain related families and genera for which the sporocyst walls have been found to be composed of a number of plates which are joined by a specialized structure (2, 12 13 16 17 22, 23)

In our experience, the sporocyst wall seems, in its resistance to chemical agents, to be similar to the oocyst wall and thus only sporocysts, the walls of which had been broken, were properly fixed and embedded. Why these parasites should be doubly protected against chemical interference is unclear since the oocyst wall alone should provide sufficient protection in the external environment However this circumstance may be of evolutionary significance because a protective sporocyst wall will be a necessity for species which produce sporocysts which are not protected by an oocyst wall

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THE EFFECT OF NALIDIXIC ACID RIFAMPICIN AND CHLORAMPHENICOL ON THE SYNTHESIS OF PHOSPHOLIPASE C IN *BACILLUS CEREUS*

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Valle, K. J and Prydz, H The effect of nalidixic acid, rifampicin and chloramphenicol on the synthesis of phospholipase C in *Bacillus cereus* Acta path microbiol scand Sect B, *86* 25–28, 1978

The effect of nalidixic acid, rifampicin and chloramphenicol on the synthesis of phospholipase C (EC 3 1 4 3) has been studied in washed *Bacillus cereus* cells resuspended in nutrient broth. In the absence of inhibitors, the synthesis showed a biphasic pattern No synthesis or release of enzyme was found in the presence of chloramphenicol When rifampicin was added, phospholipase C synthesis continued for 10–15 min Nalidixic acid, in concentrations which inhibited DNA synthesis completely permitted the synthesis of phospholipase C at the same rate and for a similar length of time as rifampicin

Key words phospholipase C, exoenzyme synthesis, *Bacillus cereus*

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The synthesis of extracellular protease or α-amylase in washed *Bacillus amyloliquefaciens* cells suspended in fresh medium shows a biphasic pattern, whereas other extracellular enzymes such as RNase show other patterns (3 4) The intracellular level of protease is very low (10), and transport and release of preformed enzyme account for only a small part of the first phase of increased enzyme activity most of which is chloramphenicol-sensitive de novo synthesis of protease on preformed mRNA templates This rifampicin-resistant production lasts for 60–80 min A rifampicin-resistant synthesis of about 40 min duration was also found for two other extracellular enzymes from the same strain (RNase and α-amylase) (6) This implies either (or both) an increased stability of mRNA for these extracellular enzymes in this strain, or an increased size of the pool of the relevant mRNA species The biphasic pattern of increased protease synthesis was assumed to be due to repression by the amino acids present in the medium. As the amino acids were metabolized, protease mRNA transcription was supposedly derepressed

The study of a different exoenzyme in another strain of Bacillus might provide information concerning the generality of these observations. For this purpose we chose phospholipase C (EC 3 1 4 3) (PLC) from *Bacillus cereus*.

MATERIALS AND METHODS

Bacillus cereus ATCC 10987 was stored on Brain Heart Infusion agar (Oxoid) Liquid cultures were grown on a shaker at 150 rev/min and 37° C, using the low molecular fraction of nutrient broth (Oxoid) obtained by dialysis (13) Growth was monitored at 600 nm

Washed cell suspensions Liquid cultures (150 ml) were harvested after about 3 h at an absorbancy of 0 80–0 85, i e in the late logarithmic phase, when the rate of synthesis of phospholipase C was highest (13) The cells in 18 ml samples were harvested on 0.22 μ Millipore filters, washed with 10 ml prewarmed fresh medium and transferred to Erlenmeyer flasks with 15 or 20 ml fresh medium to which the relevant isotopes and inhibitors were added The whole procedure was carried out at 37° C and required less than 3 min.

Isotope labelling (U-^{14}C)-L-leucine (50 μCi/ml, specific activity 348 Ci/mol) (200 μl) and 5,6-^{3}H-uridine

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Isotope labelling (U-^{14}C)-L-leucine (50 μCi/ml, specific activity 348 Ci/mol) (200 μl) and 5,6-^{3}H-uridine

(1 mCi/ml specific activity 41 Ci/mmol) (12 µl) (from the Radiochemical Centre, Amersham) were added to 15 ml aliquots of washed resuspended cells.

Assays One ml samples were withdrawn for determination of absorbancy at 600 nm and afterwards centrifuged (10 000 G/10 min/4° C). The pellets were used for determination of DNA and isotope incorporation. The PLC activity was measured in the supernatants by inactivation of a standard suspension of tissue thromboplastin (2) as described earlier (13 14) One enzyme unit inactivated one unit of tissue thromboplastin per min at 37° C

Where the enzyme activity was high, the supernatants were diluted with veronal-buffered saline (7) containing 1 mM $ZnCl_2$ Chloramphenicol (Sigma) rifampicin (Lepetit) and nalidixic acid (Sigma) did not influence the assay system in the concentrations used

The cell pellets were extracted with 2 ml cold 0.5 M perchloric acid (PCA) for 15 min at 2° C and centrifuged The sediments were washed four times in cold 0 5 M PCA. The radioactivity of the washing fluids was determined. For isotope counting, the final pellets were dissolved in 1 ml 0 5 M NaOH for 48 h at 37 C in stoppered tubes. Three samples of 0 1 ml from each hydrolysate were mixed with 10 ml Instagel (Packard) and counted in a Packard Tricarb 3375 Scintillation Counter The quenching effect was found to be insignificant.

For DNA determination, the solutions were reacidified with cold HCl reprecipitated with an equal volume of cold 0 5 M perchloric acid and centrifuged after 2 h at 4° C The final pellets were extracted with 1 ml 0 5 M PCA at 90° C for 20 min DNA was measured according to *Burton* (5), with deoxyribose as standard Protein was measured by the method of *Lowry et al* (9) with bovine serum albumin (Sigma) as standard Monospecific antibodies to purified PLC (8) were raised and purified as described (12)

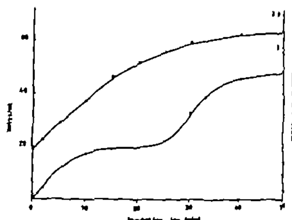


Fig 1 Growth and synthesis of phospholipase C in *Bacillus cereus*
——●—— Absorbancy at 600 nm
——▲—— Phospholipase C activity (units/ml)

RESULTS AND DISCUSSION

After filtration and resuspension of cells harvested in late log phase, the increase of PLC activity in the medium followed a biphasic pattern (Fig 1). The first phase lasted about 10–15 min and was followed by a second period of increased activity which usually began about 25–30 min after resuspension of the cells. The maximal increase of PLC activity was similar in the two phases (1–2 units $^{-1}$ min^{-1}). The half lives of the PLC activity present in supernatants from cultures harvested at various times after resuspension were similar (about 20 min) so that differences in turnover did not explain the observation.

The intracellular enzyme activity was tested in homogenates of washed bacterial cells harvested at the higher PLC synthesis rate The cells were broken by homogenization in a Teflon-glass Potter Elvehjem homogenizer by sonication or by alumina grinding. No PLC activity or only traces were found, and no antigenic material was detectable with anti PLC antibodies in immunodiffusion or immunoelectrophoresis when extracts of 10^9–10^{10} cells/ml were prepared.

At high concentrations of chloramphenicol (200–400 µg/ml) no increase in PLC activity was seen (Fig 2) Such concentrations immediately reduced ^{14}C-leucine incorporation into protein to about 1–2 per cent of the amount in control cultures The cause of the 10 min lag in leucine incorporation in the control cells is unknown Incorporation of ^{3}H uridine into RNA was reduced after 10 min (Fig. 3) Altogether these results indicated that the first phase of PLC increase was not caused by release of preformed enzyme present intracellularly at the

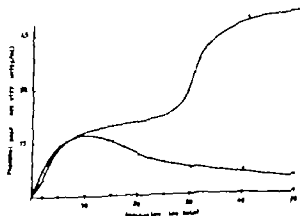


Fig 2 Synthesis of phospholipase C in the presence of rifampicin or chloramphenicol
——△—— Control culture
——▲—— Rifampicin (60 µg/ml)
——●—— Chloramphenicol (400 µg/ml)

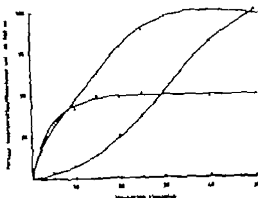


Fig. 3. Incorporation of ^{14}C-leucine and ^{3}H-uridine into *Bacillus cereus* in the presence of chloramphenicol

———△——— ^{3}H-uridine incorporation in control culture
———▲——— ^{3}H-uridine incorporation in presence of chloramphenicol (400 µg/ml)
———○——— ^{14}C-leucine incorporation in control culture
———●——— ^{14}C-leucine incorporation in presence of chloramphenicol (400 µg/ml)

For ^{3}H-uridine 100 % is 5691 cpm/absorbancy unit
For ^{14}C leucine 100 % is 1420 cpm/absorbancy unit

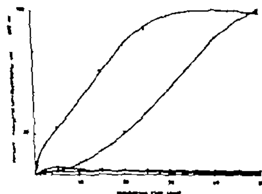


Fig. 4. Incorporation of ^{14}C-leucine and ^{3}H-uridine in the presence of rifampicin

———△——— ^{3}H-uridine in control cultures
———————— ^{3}H-uridine in presence of rifampicin (60 µg/ml)
———○——— ^{14}C-leucine in control cultures
———●——— ^{14}C-leucine in presence of rifampicin (60 µg/ml)

For ^{3}H-uridine 100 % is 10000 cpm/absorbancy unit
For ^{14}C-leucine 100 % is 3400 cpm/absorbancy unit

start of incubation, and that therefore excretion of PLC is closely coupled in time with its biosynthesis.

The addition of rifampicin (40–100 µg/ml) reduced the incorporation of ^{3}H-uridine to 52 per cent after 2 min and to about 10 per cent after 5 min (Fig. 4). However the increase in PLC activity was similar to that in the control cultures for 10–15 min (Fig. 2), whereas ^{14}C-leucine incorporation declined within 5 min (Fig. 4). After 10–15 min, the rate of PLC activity increase declined rapidly and the secondary phase of the increase was prevented completely (Fig. 2). The first phase of the increase in PLC activity was thus sensitive to chloramphenicol and resistant to rifampicin, suggesting that *de novo* synthesis of PLC took place on preformed mRNA templates, whereas the second phase required the synthesis of new mRNA.

Delayed addition of rifampicin (20–25 min after resuspension and start of incubation) resulted in the same duration and extent of PLC activity increase as immediate addition.

In preliminary experiments, 70–80 µg/ml of nalidixic acid completely inhibited DNA replication. At this concentration, the effect on PLC synthesis was very similar or identical with that of rifampicin, i.e. permitting the first and preventing the second phase of PLC activity increase.

The synthesis of PLC in *B. cereus* thus resembled that of the various exoenzymes in *B. amyloliquefaciens* (4, 6), in that the late log phase cells contained a pool of mRNA permitting the synthesis of exoenzymes for long periods of time in the presence of rifampicin. Also, the biphasic pattern of PLC activity increase resembled the pattern of the extracellular protease in *B. amyloliquefaciens* (3) and the α-amylase in *B. subtilis* (15). The amino acid level in the growth medium does not seem to be related to the synthesis of PLC as in the case of the protease (3), since the rate of synthesis of PLC increases in media rich in amino acids. We have confirmed the absence of a detectable intracellular pool of enzymatically or antigenically active protein, and the results are consistent with a close coupling between synthesis, excretion and the acquirement of an active configuration, as postulated by *May & Elliott* (11).

The present results are partly different from the data of *Both et al.* (3) for the protease mRNA, the pool of which was exhausted during growth of resuspended bacteria at high amino acid concentrations, but similar to their observations of a significant pool of exoenzyme mRNA also in phase 2 when the cells were grown at low amino acid concentrations. The most likely explanation for the rifampicin-resistant PLC synthesis is the existence of a pool of PLC-mRNA. The biphasic synthesis

pattern is possibly due to a temporary block in RNA synthesis through changes in the levels of cyclic nucleotides caused by the manipulation of the cells during filtration washing and resuspension. This block would have to be partial and specific, since no lag in bacterial growth or RNA synthesis is observed.

The finding (3 15 present paper) that three different exoenzymes in three different strains of Bacillus have a biphasic synthesis pattern in common is consistent with other observations using mutants in *B subtilis* (1 17 18) or a peptide inhibitor of exoenzyme synthesis (*16*) This would suggest that there is a common regulation at some step of exoenzyme synthesis in Bacillus strains.

Since the extracellular enzymes and toxins are important for the pathogenetic effects of the *Bacillaceae* further studies of this regulation may be of interest.

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BINDING OF AGGREGATED IgG IN THE PRESENCE OF FRESH SERUM BY GROUP A STREPTOCOCCI PRODUCING PHARYNGEAL INFECTION

POSSIBLE CONNECTION WITH TYPES FREQUENTLY INVOLVED IN ACUTE NEPHRITIS

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Christensen, P., Sjöholm, A. G., Holm, S. E., Hovelius, B. & Mårdh, P. A. Binding of aggregated IgG in the presence of fresh serum by group A streptococci producing pharyngeal infection: possible connection with types frequently involved in acute nephritis. Acta path. microbiol. scand. Sect. B, 86: 29–33, 1978.

109 streptococcal strains, belonging to diverse serological groups and types, were investigated as regards their capacity to bind IgG aggregates in the presence of fresh serum. Strains capable of such binding were not found in groups B, C, D, E, G, L, M or N. Such binding was restricted to a few types of group A streptococci: the potentially nephritogenic types 2, 6 and 12, and four strains belonging to types M 39, M 46 and M 22 or M 62, the nephritogenic capacity of which is unknown. Two of five strains isolated from patients with acute post-streptococcal glomerulonephritis (AGN) and 19/28 type T 12, 8OR strains isolated during an epidemic in a kindergarten with associated cases of AGN were found to bind aggregates. The findings suggest a possible association between capacity to bind aggregates in the presence of serum and the serological types of group A streptococci involved in acute nephritis following pharyngeal infection.

Key words: Binding of aggregated IgG; group A streptococci; fresh serum; acute nephritis; pharyngeal infection.

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The association (*Rammelkamp et al.* 1952, *Rammelkamp & Weaver* 1953) between infection with group A streptococci of certain M types and acute post-streptococcal glomerulonephritis (AGN) appears to be well established (*Rammelkamp* 1964). However, despite extensive investigation during the past two decades (see *Gutzberg* 1972), the nephritogenic potential of these streptococci remains unexplained.

The etiological importance in AGN of M type 12 streptococci infecting the throat is well recognized (*Rammelkamp* 1964). Recently it was found (*Christensen et al.* 1977) that binding of aggregated IgG by M+ve type 12 streptococci was high in the presence of fresh serum, while M−ve type 12 streptococci and M+ve or M−ve variants of two other types showed low uptake of IgG aggregates under the test conditions applied. The reaction was shown to be complement dependent, requiring the presence in serum of at least C1 and C4.

The present investigation included several streptococcal strains of diverse serological types and groups, in order to evaluate further a possible relationship between the nephritogenic potential of individual strains and the capacity to bind aggregated IgG in the presence of fresh serum.

pattern is possibly due to a temporary block in RNA synthesis through changes in the levels of cyclic nucleotides caused by the manipulation of the cells during filtration, washing and resuspension. This block would have to be partial and specific, since no lag in bacterial growth or RNA synthesis is observed

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TABLE 2 *Uptake of Radio-labelled Aggregated IgG in the Presence of Fresh Serum by Group A Streptococci Isolated from Patients with Acute Post-streptococcal Nephritis or During an Epidemic Outbreak of Nephritis*

Strains		Uptake of 125I-labelled aggregated IgG (per cent of 1 μg added)
Strains isolated from patients with acute nephritis	type T 6 5390/68	40
	type T 12 589/69	0
	type T 12 865/66	0
	type T 12 1070/64	0
	type T 12 3541/64	52
Type T 12, SOR– strains isolated from one epidemic outbreak	9 strains	0
	6 strains	30–40
	10 strains	40–50
	3 strains	50–60

patients with acute nephritis were tested. Two of these, a T 6 and a T 12 strain, showed high uptake of aggregated IgG in the presence of fresh serum (Table 2).

Strains Isolated during an Epidemic in a Kindergarten. 28 strains, selected at random from T 12 strains isolated from the children in a kindergarten during an epidemic of streptococcal sore throat with transient light nephritis (Bygren et al. 1977) were tested for reactivity with IgG aggregates in the presence of fresh serum. 19 showed high uptake of aggregated IgG (Table 2).

Strains Isolated from Patients without Acute Post-streptococcal Nephritis. Twenty-five strains were selected at random from throat swab specimens obtained from patients with upper respiratory tract infection but without pathological urinary microscopy findings. Thirteen bound aggregated IgG. Judging from the results of T typing and SOR and NADse tests, the strains probably belonged to the following M types: 10 to type M 12, 2 to type M 22 or M 62, and 1 strain to M 2. Of the non-reactive strains, only one was T type 12 (probably M 12), while the others belonged to other types or else were non-T-typable (Table 3).

TABLE 3 *Uptake of Radio-labelled Aggregated IgG in the Presence of Fresh Serum by Group A Streptococci Isolated from Patients without Acute Post-streptococcal Nephritis*

No. of Strains	T-type	Serum Opacity Reaction	NADse production	Implicated[1] M-types	Uptake of 125I-labelled aggregated IgG (percentage of 1 μg added)
1	2	+	+	M 2	52
1	2	+	–	M 2	0
2	2	+	+	M 2	0
1	4	+		M 4, 28, 48, 60	0
10	12	–	+	M 12	27 – 59 (mean 47)
1	12	–	+	M 12	0
2	12			M 22, 62	50 – 52
1	12		+	M 22, 62	0
1	28			M 4, 28, 48, 60	0
1	not typable	–	–		0
2	not typable		+		0

[1] see *Havem & Christensen* (1977). The relation of M 62 to T 12, SOR– was described by *Maxted et al.* (1974).

MATERIALS AND METHODS

Reference Strains

The following strains were used

Group A streptococci M 1 (8198) T 1 (SF130), M 2 (8322) M 3 (100064), M 4 (SS 241) M 5 (100065) M 6 (8302), M 8 (8324), M 9 (100067), M 11 (100068), M 12 (M+ve and M–ve variants, 1130), M 15 (100070) M 17 (8304) M 18 (EF 1896) M 22 (EF 1950), M 27 (EF 1913) M 28 (EF 1899) M 31 (8171) M 33 (EF 1926), M 36 (EF 1928), M 38 (EF 1921), M 39 (EF 1922), M 41 (EF 1924) M 42 (EF 1927) M 44 (8231) M 46 (EF 1925) and M 48 (EF 1918)

Group B streptococci type Ia (O 90S), Ib (H 36B), Ic (A 909), II (18 RS 21) III (D 136C) and the O 90R variant.

Group C streptococci T 20 J (EF 1912), K 64-0-13 (EF 1905), K 39 (EF 1904), 7 J (EF 1860), SPA 1 (EF 1854), SPA 2 (1853).

Group D streptococci 10085 (EF 1852), C 1 (EF 1919), C 3 (EF 1920).

Group G streptococci D 166B (EF 1969) 16 J (EF 1859), F 68 (EF 1911), 51/512.

Streptococci belonging to other groups group E. K 129 (EF 1948), group L. D 167 A(EF 1941), group M D 168 A(EF 1943), group N (EF 1955).

Strains Isolated from Patients with Acute Post streptococcal Glomerulonephritis

The following group A streptococci, isolated from patients with acute post-streptococcal nephritis, were kindly supplied by Statens Seruminstitut, Copenhagen, Denmark type T 6 (5390/68), types T 12, strains 589/69 865/66 1070/64 and 3541/64

Strains Isolated during an Epidemic in a Kindergarten

Group A streptococci 28 strains, all belonging to type T 12 and with a negative serum opacity reaction (SOR–) (*Maxted et al* 1974) were selected at random from among the strains isolated during a recent epidemic in a kindergarten. 44 of 84 children were infected with this strain, as revealed by throat swab cultures, and 8 of the infected children showed slight signs of acute glomerulonephritis (urine microscopy cylinders, haematuria, proteinuria) (*Bygren et al* 1977) None of the children were clinically affected, apart from the pathological urinary affection which subsided after 2 weeks One adult female, a relative to one of the children, developed a typical AGN with transient hypocomplementaemia

Other Group A Streptococci

Twenty five strains of group A streptococci were selected at random from throat swab cultures from patients with upper respiratory tract infection but without pathological urinary microscopy findings. The strains were T-typed and tested for opacity factor and nicotinamide adenine dinucleotide glycohydrolase (NADse), as described previously (*Ivarsson & Christensen* 1977)

Test for Uptake of ¹²⁵I Labelled Aggregated IgG in the Streptococci

The test conditions have been described previously (*Christensen et al* 1977) In brief 1 μg ¹²⁵I labelled IgG aggregates (50 μl) were incubated with 50 μl of fresh serum at 37° C for 30 min. 200 μl of a standard suspension of the test strain was then added, followed by incubation at 22° C for 30 min. The bacteria were separated by centrifugation and after appropriate corrections, the radioactivity bound in the bacterial pellet was expressed as percentage of the radioactivity added

RESULTS

Binding of Radio-labelled Aggregated IgG to Streptococci in the Presence of Fresh Serum

Reference Strains. 28 group A streptococci, representing 26 different M types, were tested. Of these only 3 strains showed high uptake of aggregated IgG in the presence of fresh serum These were M 12 M + ve, M 39 and M 46 which bound 58 39 and 44 per cent of the 1 μg aggregates added respectively None of the other group A streptococci or the strains belonging to other serological groups bound any detectable amounts of aggregated IgG (Table 1).

Strains Isolated from Patients with Acute Post streptococcal Nephritis Five strains isolated from

TABLE 1 *Uptake of Radio-labelled Aggregated IgG by Various Reference Strains of Streptococci in the Presence of Fresh Serum*

Strains	Uptake of ¹²⁵I-labelled aggregated IgG (percentage of 1 μg added)
Group A (25 strains) M 1 T 1 M 2, M 3 M 4 M 5 M 6 M 8 M 9 M 11 M 12 (M–ve), M 15 M 17 M 18 M 22, M 27 M 28 M 31 M 33 M 36 M 38 M 41 M 42, M 44 M 48	0
M 12 (M + ve)	58
M 39	39
M 46	44
Other groups B (6 strains), C (6 strains), D (3 strains), G (4 strains), E (1 strain) L (1 strain) M (1 strain), N (1 strain)	0

TABLE 2 *Uptake of Radio-labelled Aggregated IgG in the Presence of Fresh Serum by Group A Streptococci Isolated from Patients with Acute Post streptococcal Nephritis or During an Epidemic Outbreak of Nephritis*

Strains		Uptake of 125I-labelled aggregated IgG (per cent of 1 μg added)
Strains isolated from patients with acute nephritis	type T 6 5390/68	40
	type T 12 589/69	0
	type T 12 865/66	0
	type T 12 1070/64	0
	type T 12 3541/64	52
Type T 12, SOR− strains isolated from one epidemic outbreak	9 strains	0
	6 strains	30–40
	10 strains	40–50
	3 strains	50–60

patients with acute nephritis were tested. Two of these, a T 6 and a T 12 strain, showed high uptake of aggregated IgG in the presence of fresh serum (Table 2).

Strains Isolated during an Epidemic in a Kindergarten. 28 strains, selected at random from T 12 strains isolated from the children in a kindergarten during an epidemic of streptococcal sore throat with transient light nephritis (Bygren *et al.* 1977) were tested for reactivity with IgG aggregates in the presence of fresh serum. 19 showed high uptake of aggregated IgG (Table 2).

Strains Isolated from Patients without Acute Post streptococcal Nephritis. Twenty-five strains were selected at random from throat swab specimens obtained from patients with upper respiratory tract infection but without pathological urinary macroscopy findings. Thirteen bound aggregated IgG. Judging from the results of T typing and SOR and NADase tests, the strains probably belonged to the following M types: 10 to type M 12, 2 to type M 22 or M 62, and 1 strain to M 2. Of the non-reactive strains, only one was T type 12 (probably M 12), while the others belonged to other types or else were non-T-typable (Table 3).

TABLE 3 *Uptake of Radio-labelled Aggregated IgG in the Presence of Fresh Serum by Group A Streptococci Isolated from Patients without Acute Post-streptococcal Nephritis*

No. of Strains	T-type	Serum Opacity Reaction	NADase production	Implicated 1) M-types	Uptake of 125I-labelled aggregated IgG (percentage of 1 μg added)
1	2	+	+	M 2	52
1	2		−	M 2	0
	2			M 2	0
1	4		+	M 4, 28, 48, 60	0
10	12	−	+	M 12	27 – 59 (mean 47)
1	12	−	+	M 12	0
2	12		+	M 22, 62	50 – 52
1	12			M 22, 62	0
1	28		+	M 4, 28, 48, 60	0
3	not typable	−	−		0
	not typable				0

1) see Bartram & Christensen (1977). The relation of M 62 to T 12, SOR− was described by Maxted et al. (1974)

DISCUSSION

In the present investigation, 50 streptococcal reference strains of various serological types and groups were studied with regard to their capacity to bind aggregated IgG in the presence of fresh serum. Among the reference strains, reactivity was found to be restricted to three group A strains belonging to M types 12 39 and 46 Two strains isolated from patients with AGN one type T 6 and one type T 12 strain, also showed high uptake of IgG aggregates in the presence of fresh serum as did 19/28 type 12 strains isolated during an epidemic outbreak of sore throat with associated cases of AGN In all 109 strains were tested and high uptake was found in 35 strains of group A streptococci restricted to a few types. the potentially nephritogenic types 2 6 and 12 and four strains belonging to M 39 M 46 and M 22 or M 62 the nephritogenic capacity of which is not known (see below). The results were thus consistent with an association between serological types frequently connected with nephritogenicity and capacity to bind aggregates in the presence of fresh serum as far as the strains infecting the human throat are concerned. Studies of "impetigo strains" were not included in the present investigation.

Although the nephritogenicity of group A streptococci is closely associated with certain M types (*Rammelkamp* 1964), it is difficult to assess this ability to produce nephritis in any single strain. Host factors such as varying susceptibility and possibly acquired immunity to AGN might affect the frequency of renal damage in an infected population. Furthermore, strains subjected to *in vitro* investigations might have altered their cell wall structure during infection (see *Rothbath & Watson* 1948) or perhaps become changed during the subculture procedure in the laboratory. For example, it cannot be excluded, on the basis of the results of the present investigation, that recently isolated strains might be better able to bind

TABLE 4 *Binding of Aggregated IgG in the Presence of Fresh Serum Compared with Current Knowledge on the Nephritogenic Capacity of Group A Streptococcal Types*

	Strains investigated	Number of strains tested	Number of strains binding aggregated IgG in the presence of fresh serum
Possibly nephritogenic strains	types of group A streptococci associated with acute nephritis (types 2 4 6 and 12)[a]	27	15 (56%)[e]
	types of group A streptococci concerning the nephritogenicity of which conflicting reports exists (types 1 and 3)[b]	3	0
Probably not nephritogenic strains"	other group A streptococcal types, known not to be associated with acute nephritis or not sufficiently investigated with respect to nephritogenicity[c] T types connected with more than one M type and non-typable freshly isolated strains[d]	28	4 (14%)[e]

Types of group A streptococci isolated from patients with acute nephritis, arranged according to frequency of isolation from the throat types 12 1 4 49 25 and 3 (*Rammelkamp* 1964) Type 2 was associated recently with acute nephritis (*Anthony et al* 1974) The strains isolated from the kindergarten epidemic are not included

b See remark However *Rammelkamp et al* (1952) and *Rammelkamp & Weaver* (1953) failed to observe acute nephritis following a large number of untreated pharyngeal infections with types 1 and 3

c *Rammelkamp et al* (1952) and *Rammelkamp & Weaver* (9153) failed to observe acute nephritis following a large number of untreated pharyngeal infections with types 5 19 and 24

d See Table 3

Significantly more types of group A streptococci associated with acute nephritis bound aggregated IgG in the presence of fresh serum than probably not nephritogenic strains ($p < 0.05$ Chi Square Test)

aggregated IgG in the presence of fresh serum than subcultured strains. With these reservations in mind it can be concluded from extensive studies performed by *Rammelkamp et al.* (1952) and *Rammelkamp & Weaver* (1953) that the nephritogenic potential of type 12 group A strains is outstanding among the streptococci infecting the human throat, and that types 4, 6 and 25 can also exert a nephritogenic influence. Nevertheless, sporadic cases provoked by other types have also been described. It has been claimed recently that type 2 group A streptococci are potentially nephritogenic during throat infections (*Anthony et al.* 1974). As regards types 39 and 46, which bound aggregated IgG in the presence of fresh serum, the strains were isolated from the same patient in May 1941 at the Rockefeller Institute Hospital. Little is known regarding these types, and nothing is known about their nephritogenic capacity (*R. C. Lancefield*, personal communication). The current knowledge concerning the association of nephritogenicity to certain types and the capacity to bind aggregated IgG in the presence of fresh serum is summarized in Table 4.

As discussed above, the difficulties encountered in assessing the nephritogenic capacity of any single strain are not the only reason why care must be exercised in the interpretation that binding of IgG aggregates in the presence of fresh serum is a property of nephritogenic strains. A causal relationship between nephritogenicity and ability to bind aggregates under the test conditions described remains speculative. It is of obvious interest that binding of aggregates is influenced at least by complement factors C 1 and C 4 (*Christensen et al.* 1977) since involvement of the complement system is a typical feature of AGN (*Michael et al.* 1966).

To summarize, the findings in the present investigation were consistent with an association between serological types of group A streptococci frequently connected with nephritogenicity and an ability to bind IgG aggregates in the presence of fresh serum among the streptococci. Further studies are required to elucidate the possible connection between nephritogenicity and binding of IgG aggregates.

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DISCUSSION

In the present investigation, 50 streptococcal reference strains of various serological types and groups were studied with regard to their capacity to bind aggregated IgG in the presence of fresh serum. Among the reference strains, reactivity was found to be restricted to three group A strains belonging to M types 12, 39 and 46. Two strains isolated from patients with AGN, one type T 6 and one type T 12 strain, also showed high uptake of IgG aggregates in the presence of fresh serum, as did 19/28 type 12 strains isolated during an epidemic outbreak of sore throat with associated cases of AGN. In all 109 strains were tested and high uptake was found in 35 strains of group A streptococci, restricted to a few types, the potentially nephritogenic types 2, 6 and 12, and four strains belonging to M 39, M 46 and M 22 or M 62, the nephritogenic capacity of which is not known (see below). The results were thus consistent with an association between serological types frequently connected with nephritogenicity and capacity to bind aggregates in the presence of fresh serum, as far as the strains infecting the human throat are concerned. Studies of "impetigo strains" were not included in the present investigation.

Although the nephritogenicity of group A streptococci is closely associated with certain M types (*Rammelkamp* 1964), it is difficult to assess this ability to produce nephritis in any single strain. Host factors such as varying susceptibility and possibly acquired immunity to AGN might affect the frequency of renal damage in an infected population. Furthermore, strains subjected to *in vitro* investigations might have altered their cell wall structure during infection (see *Rothbath & Watson* 1948), or perhaps become changed during the subculture procedure in the laboratory. For example it cannot be excluded, on the basis of the results of the present investigation, that recently isolated strains might be better able to bind

TABLE 4. *Binding of Aggregated IgG in the Presence of Fresh Serum Compared with Current Knowledge on the Nephritogenic Capacity of Group A Streptococcal Types*

	Strains investigated	Number of strains tested	Number of strains binding aggregated IgG in the presence of fresh serum
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[a] Types of group A streptococci isolated from patients with acute nephritis, arranged according to frequency of isolation from the throat: types 12, 1, 4, 49, 25 and 3 (*Rammelkamp* 1964). Type 2 was associated recently with acute nephritis (*Anthony et al.* 1974). The strains isolated from the kindergarten epidemic are not included.

[b] See remark[a]. However *Rammelkamp et al.* (1952) and *Rammelkamp & Weaver* (1953) failed to observe acute nephritis following a large number of untreated pharyngeal infections with types 1 and 3.

[c] *Rammelkamp et al.* (1952) and *Rammelkamp & Weaver* (9153) failed to observe acute nephritis following a large number of untreated pharyngeal infections with types 5, 19 and 24.

[d] See Table 3.

[e] Significantly more types of group A streptococci associated with acute nephritis bound aggregated IgG in the presence of fresh serum than probably not nephritogenic strains ($p < 0.05$; Chi Square Test).

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SYNTHETIC DISACCHARIDE-PROTEIN ANTIGEN FOR PRODUCTION OF SPECIFIC O2 ANTISERUM FOR IMMUNOFLUORESCENCE DIAGNOSIS OF SALMONELLA

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Svenungsson, B. & Lindberg, A. A. Synthetic disaccharide-protein antigen for production of specific O2 antiserum for immunofluorescence diagnosis of salmonella. Acta path. microbiol. scand. Sect. B, 86: 35–40, 1978.

Antisera from rabbits immunized with the synthetic disaccharide paratose 1→3 mannose, representative of *Salmonella* O-antigen 2, covalently linked to bovine serum albumin (BSA), were used in indirect immunofluorescence studies for the identification of *Salmonella* serogroup A (O-antigen 1,2,12) bacteria. Among 1311 enteric bacteria tested, 497 were *Salmonella*. The anti-paratose 1→3 mannose-BSA serum identified correctly all the 63 serogroup A strains tested. No positive reactions were recorded among 1248 strains representing *Salmonella* other than serogroup A, *E. coli*, *Shigella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Pseudomonas*, *Acinetobacter*, *Vibrio*, *Yersinia* and *Bacteroides*. The study illustrates the high specificity of the antiserum elicited by immunization with the synthetic disaccharide-protein immunogen.

Key words: Salmonella; immunofluorescence diagnosis; synthetic disaccharide-protein; antiserum.

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A rapid and accurate diagnosis of *Salmonella* bacteria is of great importance, both in human and veterinary health care and food control. In this respect, the immunofluorescence technique is an alternative to conventional culture methods, but lack of specificity of antibody preparations used has often limited its applicability (4,5,8,16,21,24).

The serogroup designation of *Salmonella* bacteria is based on the O-antigen make-up. The O-antigenic specificity resides in the polysaccharide chain of the lipopolysaccharide, a structural component of the outer membrane of the bacterial cell envelope (19). The structure of the O-antigenic polysaccharide chain in *Salmonella* serogroup A, B, C2, C3, D and E 1–4 has been determined previously (9–15, 19). In *Salmonella* serogroup A, B and D the chain consists of repeating units of oligosaccharides, where the group specific antigenic determinants can be represented by disaccharides: in serogroup A paratose 1→3 mannose, in serogroup B abequose 1→3 mannose, and in serogroup D tyvelose 1→3 mannose, representing the O-antigens 2, 4 and 9 respectively (Fig. 1). These antigenic determinants have been synthesized (1) and linked to bovine serum albumin (BSA) (via the synthesis of the phenylisothiocyanate disaccharide glycoside) as an immunogenic carrier (7,20). Antisera from rabbits immunized with the synthetic disaccharide-protein conjugates abequose 1→3 mannose-BSA (AM-BSA) and tyvelose 1→3 mannose-BSA (TM-BSA) were shown to be highly specific when tested in passive haemagglutination and complement-mediated bactericidal tests (7). In a previous paper we were able to demonstrate the high specificity of the anti AM-BSA and TM-BSA sera when used for immunoflu-

TABLE 1 *ELISA Antibody Titres in Rabbits Immunized with Paratose 1→3 Mannose – BSA*

Antiserum	Absorbance at 400 nm/100 min, serum diluted 10^{-4} Antigens		
	S paratyphi A var durazzo (O2, 12_1 12_3)	*S typhimurium* LT2 (O4 5 12_2)	*S typhi* T2 (O9 12_1 12_3)
PM–BSA	23 9	0 23	0 31
Preimmunization	<0 10	0 12	0 10

ELISA using lipopolysaccharides (LPS) from *S paratyphi* A var durazzo (2 12_1 12_3) *S typhimurium* LT2 (4 5 12_2) and *S typhi* T2 (9 12_1, 12_3) (Table 1) Only in systems where the *S paratyphi* A LPS was used as antigen could a significant increase in titre be observed between sera collected before and after immunization. Thus, in this respect the PM BSA conjugate elicited an antibody response representative of O-antigen 2

Indirect Immunofluorescence Studies Using Known Salmonella *Strains*

Sera from the four rabbits immunized with PM BSA were titrated against a *Salmonella paratyphi* A strain (O-antigen 2 12), using the indirect immunofluorescence technique The end-point dilution value (defined as the last dilution which gave a 3+ reaction) after three immunizations varied between 1/2560 and 1/5120 (Table 2) When the sera were tested against either a *S enteritidis* strain (O-antigen 9 12) or a *S typhimurium* strain (O-antigen 4 5 12) as negative controls, no fluorescence reaction was detected with serum diluted 1/5 or more Neither preimmunization sera nor anti-BSA sera gave any fluorescence reactions at any dilution. The last dilution of the antiserum which gave a 4+

TABLE 2 *Indirect Immunofluorescence End-point Titres of Rabbits Immunized with Paratose 1→3 Mannose – BSA*

Rabbit no	End point titres	
	Day 38	Day 142
1	1/320	1/2560
	1/40	1/2560
3	1/ 560	1/2560
4	1 1280	1/5120

Rabbits were immunized with 10 µg of the disaccharide protein conjugate suspended in Freund' complete adjuvant on days 0 30 and 126

TABLE 3 *Indirect Immunofluorescence (IFL) Studies of Salmonella Using Anti-PM BSA and Factor O2 Sera*

Salmonella serogroup	No of strains	No positive in IFL PM–BSA	Factor O2
A (O1,2,12)	63	63	49
B (O1 4,5 12)	25	0	5
C (O6 7,6,8 8,20)	25	0	0
D (O1 9 1)	25	0	3
E (O3 10 3 15 3,34 1 3 19)	25	0	0

4+ and 3+ reactions were scored as positive

reaction was used as working dilution, this was usually two or three twofold dilution steps lower than the end-point titre

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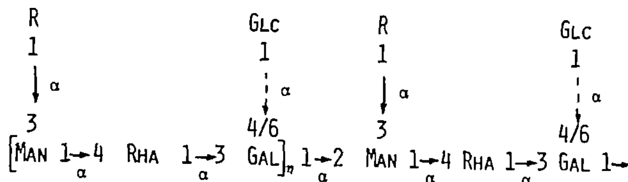


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PM-BSA	2.39	0.23	0.31
Preimmunization	<0.10	0.12	0.10

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Rabbit no	End point titres Day 38	Day 142
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Rabbits were immunized with 10 µg of the disaccharide protein conjugate suspended in Freund's complete adjuvant on day 0, 30 and 1 6

TABLE 3 *Indirect Immunofluorescence (IFL) Studies of Salmonella Using Anti PM BSA and Factor O2 Sera*

Salmonella serogroup	No of strains	No positive in IFL PM-BSA	Factor O2
A (O1,2,12)	63	63	49
B (O1,4,5,12)	25	0	5
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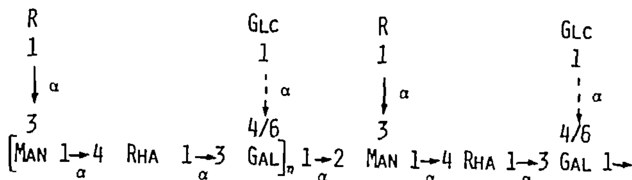


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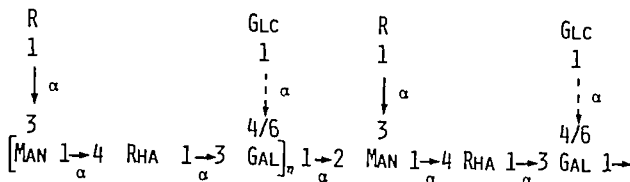


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The specificity of the anti-P3I BSA serum was considered to be high also when 667 non-*Salmonella* enteric bacteria were examined (Table 4). With the exception of a few occasional non reproducible positive reactions with *Bacteroides fragilis*, no positive isolates were found. As observed earlier with the anti-TMI BSA serum, a few *Bacteroides fragilis* strains (later shown to be encapsulated) stained with the antiserum elicited by immunization with the disaccharide-protein conjugate (23). However absorption experiments have shown that the antibodies that react with the anaerobic bacteria are not directed against the disaccharide haptens. The exact cause of the cross-reactivity observed is not known. The problems encountered in reproducing these reactions make us to believe that the antibodies concerned react with the *B. fragilis* capsule. It must be stressed that the cross-reactions with *B. fragilis* do not cause any problems in the diagnostic work as long as the immunofluorescence tests are performed on bacteria grown under aerobic conditions.

This work was supported by grants from the Swedish Medical Research Council (No 40 X-656) and the Swedish Board of Technical Development (No 74-4852 and No 75-3526). We are thankful to Professor *L. Le Minor* for supplying *Salmonella* serogroup A strains, and to Mrs *A. C. Palmgren* for excellent technical assistance.

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Indirect Immunofluorescence Studies Using Known Non Salmonella *Enteric Bacteria*

The specificity of the anti PM BSA serum was subsequently tested by examining 667 non-*Salmonella* enteric bacteria. The strains came either from the strain collection or were fresh isolates representing common microorganisms in the gut. 475 of the strains examined belonged to *Enterobacteriaceae* and all were negative. All strains of *Pseudomonas, Aeromonas Acinetobacter Vibrio cholerae V.enteritidis* and *V parahaemolyticus* were also negative. Out of 154 *Bacteroides fragilis* strains tested, 7 were positive in the initial test but negative when reexamined three times (Table 4) The reason for this remains unexplained.

Indirect Immunofluorescence Studies Using Unknown Enteric Bacteria from Faecal Samples

A further investigation was done on colony material from fresh faecal samples submitted for examination. The experiments were carried out as follows: –

One loopful of a suspect colony was suspended in a drop of PBS on a glass slide for immunofluorescence examination. One loopful of the same colony was inoculated simultaneously in test tubes for biochemical reactions and on agar plates for subsequent serological investigation. A total of 481 samples was examined, among which 334 *Salmonella* were found. They represented 10 different serogroups and 26 different species (Table 5). No *Salmonella* serogroup A strains were found among the samples examined. All the *Salmonella* strains were negative as were 147 strains which were not *Salmonella* (Table 5). *Proteus* species dominated among the non *Salmonella* strains.

TABLE 4 *Indirect Immunofluorescence Studies of Enterobacteria other than* Salmonella *Using Anti PM BSA Serum*

Genus/species	No of strains	No. positive in IFL[a]
E coli	237	0
Shigella	14	0
Citrobacter	12	0
Klebsiella	55	0
Enterobacter	12	0
Serratia	4	0
Proteus	132	0
Pseudomonas	25	0
Acinetobacter	3	0
Yersinia enterocolitica	2	0
Yersinia pseudotuberculosis	7	0
Vibrio cholerae	5	0
Vibrio enteritidis	3	0
Vibrio parahaemolyticus	2	0
Bacteroides fragilis	154	7[b]
Total	667	7

[a] 4 + and 3 + reactions were scored as positive
[b] All 7 strains were scored as negative when reexamined three times

TABLE 5 *Indirect Immunofluorescence Studies of Suspect* Salmonella *Bacterial Colonies*

Serogroups[a]	No of strains	No of positive in IFL[b]
B (O1 4,5 12) 7 species	143	0
C1 (O6 7) 6 species	67	0
C2 (O6 8) 3 species	8	0
D1 (O1 9 12) 1 species	33	0
E1 (O3 10) 3 species	15	0
E4 (O1 3 19) 2 species	56	0
G1 (O1 13,22) 1 species	3	0
I (O 16) 1 species	1	0
K (O 18) 1 species	5	0
O (O 35) 1 species	1	0
Non-Salmonella	147	0
Total	481	0

[a] Serogroup Classification is according to *Kauffmann* (17), the O-antigen formula is given in brackets
[b] Registration of the fluorescence reaction was as in Table 3

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Selection of key antigenic determinants which are coupled to immunogenic carriers should provide antigens which could elicit monospecific antisera at least in theory. The synthetic disaccharide paratose 1→3 mannose, representing the determinant for *Salmonella* O antigen 2, was coupled as hapten to bovine serum albumin (BSA). Antisera elicited in rabbits had high titres as determined in the ELISA assay (Table 1). The specificity of the antisera was evident in that no significant increase above preimmunization levels was seen when LPS from *S. typhimurium* LT2 (serogroup B) or *S. typhi* T2 (serogroup D) were used as antigens. The immunofluorescence studies, using the indirect technique, also demonstrated the high specificity of the anti-PM BSA serum. All 63 *Salmonella serogroup* A isolates were identified correctly. No false positives were recorded among a total of 434 *Salmonella* strains belonging to other serogroups (Tables 3 and 5).

The specificity of the anti-PM-BSA serum was compared with a conventional absorbed factor O2 serum on 163 *Salmonella* strains (Table 3). Using the factor O2 serum both false negative (22 percent) and false positive (8 percent) reactions were recorded. This illustrates the problem of obtaining specific antisera by immunization with whole bacteria followed by absorption procedures. The end-point titre of the factor O2 serum was also much lower (1/40) than the titres observed with the anti-PM BSA sera (1/2560–1/5120). It should be stressed, however that the factor O2 serum was prepared for slide glass agglutination.

The specificity of the anti-PM BSA serum was considered to be high also when 667 non-*Salmonella* enteric bacteria were examined (Table 4). With the exception of a few occasional, non reproducible positive reactions with *Bacteroides fragilis* no positive isolates were found. As observed earlier with the anti-TM BSA serum, a few *Bacteroides fragilis* strains (later shown to be encapsulated) stained with the antiserum elicited by immunization with the disaccharide-protein conjugate (23). However absorption experiments have shown that the antibodies that react with the anaerobic bacteria are not directed against the disaccharide haptens. The exact cause of the cross-reactivity observed is not known. The problems encountered in reproducing these reactions make us to believe that the antibodies concerned react with the *B. fragilis* capsule. It must be stressed that the cross-reactions with *B. fragilis* do not cause any problems in the diagnostic work as long as the immunofluorescence tests are performed on bacteria grown under aerobic conditions.

This work was supported by grants from the Swedish *Medical Research Council* (No 40 X-656) and the *Swedish Board of Technical Development* (No 74-485 and No 75-3526). We are thankful to Professor *L. Le Minor* for supplying *Salmonella* serogroup A strains, and to Mrs *A. C. Palmgren* for excellent technical assistance.

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CHEMOTYPES OF *FUSOBACTERIUM NUCLEATUM* LIPOPOLYSACCHARIDES

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Fredriksen, G & Hofstad, T Chemotypes of *Fusobacterium nucleatum* lipopolysaccharides Acta path microbiol scand Sect. B 86 41–45 1978

Lipopolysaccharides (LPS) were isolated from 20 strains of *Fusobacterium nucleatum* and examined by paper chromatography gas liquid chromatography and colorimetric methods for the presence of neutral sugars, amino sugars and 2-keto-3-deoxy-octonate (KDO) The LPS had in common glucosamine, L-glycero-D-manno-heptose, glucose and KDO The KDO content was low Galactose, rhamnose and D-glycero-D-manno-heptose were found in some strains Based on the sugar composition of the LPS, the *F nucleatum* strains could be classified into six chemotypes

Key words *F nucleatum*, lipopolysaccharides, chemotypes

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Lipopolysaccharides (LPS) isolated from *Fusobacterium nucleatum* are structured particles (9) which consist of a polysaccharide and a lipid component (13) The latter has a structure resembling the lipid A of *Salmonella* (5) The *F nucleatum* LPS are endotoxins (22,23) and possess o-antigenic specificity (14)

Glucosamine, heptose and small amounts (<1 5 per cent) of 2-keto-3-deoxy-octonate (KDO) were present in LPS from the strains ATCC 10953 Fev 1 and F1 of *F nucleatum* (13) In addition, LPS from F1 contained glucose and rhamnose, LPS from Fev 1 contained glucose and mannose, and LPS from ATCC 10953 contained galactose and mannose The results suggested a similarity in chemical composition of the polysaccharide part of LPS from *F nucleatum* and *Salmonella* However the apparent lack of glucose in one LPS and the finding of less than 1 5 per cent of KDO indicated structural dissimilarities LPS isolated from 17 other *F nucleatum* strains have therefore been examined, and the LPS from the three strains studied previously have been re-examined, using gas liquid chromatography in addition to paper chromatography and colorimetric methods.

MATERIALS AND METHODS

Strains

The origin of *F nucleatum* strains ATCC 10953 Fev 1 F1 and VPI 4355 (= ATCC 25586) have been described previously (6 12) Fifteen strains (F3 F5 to F18) were isolated from the saliva of the same number of healthy human subjects The strains were identified as *F nucleatum* on the basis of cellular and colonial morphology biochemical properties and antibiotic susceptibility patterns (21) Strain Fm-MC8 was isolated from an infected root canal of *Macaca cynomolgus* (1)

Cultural Conditions

Cultures were grown for 48 h in screw-cap bottles filled to the top with the following medium (g/l) Trypticase (Oxoid) 15 0 NaCl 5 0 KH_2PO_4 1.5 $Na_2HPO_4 \cdot 2H_2O$ 3 5 $NaHCO_3$ 0 5 yeast extract (Oxoid) 3 0 L-cysteine HCl 1 0 vitamin B_{12} 0 001 haemin 0 005 menadione 0 001 and glucose 2 5, pH 7 0 The microorganisms were harvested by centrifugation, washed twice with saline, and stored at −25° C until used

Isolation of LPS

The frozen microorganisms were crushed by five passages through a bacterial press, extracted overnight with phosphate buffered saline, pH 7 4 and defatted by washing with ethanol-diethylether and acetone (12) The acetone-dried material was extracted with phenol-water

(29) at 20° C for 15 min, and the LPS purified from the water phase by ultracentrifugation (100 000 × g for 90 min) and treatment with ribonuclease and deoxyribonuclease (7).

Paper Chromatography

Samples of LPS were hydrolysed at 100° C with 0 1 N HCl for 48 h, 2 N HCl for 6 h and 0 025 N H_2SO_4 for 30 min. The 0 1 N HCl and the 0 025 N H_2SO_4 hydrolysates were neutralized with Amberlite IRA 410 HCO_3^- form, and $Ba(OH)_2$ respectively. Acid was removed from the 2 N HCl hydrolysates by evaporation *in vacuo* in the presence of NaOH pellets. The samples were subjected to circular paper chromatography on Whatman No 1 paper with *n*-butanol pyridine: water (6 4 3 by vol.), *n*-butanol pyridine:0 1 N HCl (5 3 2 by vol) and *n*-butanol-acetic acid water (4 1 1 by vol) for separation of neutral sugars, KDO and amino sugars, respectively. The dried chromatograms were stained with silver nitrate or aniline hydrogen phthalate. The Elson-Morgan reagent (18) was used for detection of amino sugars, and the thiobarbituric acid reagent of *Warren* (25) for detection of KDO.

Gas Liquid Chromatography (GLC)

For chromatography of neutral sugars, samples were hydrolysed with 0 1 N HCl for 48 h at 100° C. The hydrolysed samples were neutralized with Amberlite IRA 410 HCO_3^- form, and the aldoses converted to alditol acetates, as described by *Sawardeker et al* (19). GLC was run in a Perkin-Elmer 900 Gas Chromatograph with a flame ionization detector and fitted with a glass column (internal diameter 0 20 cm 180 cm long) packed with 3 per cent ECNSS-M (W/W) on Gas-Chrom Q 100–120 mesh (Applied Science Laboratories, State College, Pa USA). The flow of N_2 gas was 30 ml per min, and the column injector and detector temperatures were 180 200 and 250° C respectively. D-xylose was used as an internal standard. The nature of the sugars was determined by comparison of the retention times of their alditol acetates with those of reference alditol acetates (Sugar Mix 1 and Sugar Mix 2 Supelco Inc. Bellefonte, Pa USA). LPS from *Salmonella typhi* strain 0901 (Difco Laboratories Detroit, Mich USA), treated as indicated, were used as reference substance for L-*glycero*-D-*manno*-heptose.

For chromatography of amino sugars, samples of LPS were hydrolysed in 2 N HCl at 100° C for 6 h in an atmosphere of N_2. The hydrolysed samples were neutralized by addition of Na_2CO_3 followed by $NaHCO_3$. Alditol acetates were prepared as described by *Niedermeier* (16), but acetic acid was used instead of HCl for decomposition of sodium borohydride. The specimens were analysed in the Perkin-Elmer gas chromatograph on a glass column packed with Poly A 103 (17) on AW DMCS 100–120 mesh (Supelco Inc) The flow of gas (N_2) was 30 ml per min, and the column, injector and detector temperatures were 200 225 and 250° C, respectively. D-glucosamine HCl and D-galactosamine HCl were used as [illegible]ence substances and D-mannosamine HCl as internal standard.

Colorimetric Determinations

The presence of KDO was investigated by the thiobarbituric acid method (28). Samples of LPS were hydrolysed with 0 02 N H_2SO_4 for 20 min at 100° C, or with 0 1 N HCl as indicated under "Paper chromatography". The optical density was examined in the range of 500 to 600 nm and a peak or a shoulder at 550 nm was recorded as a positive reaction. Heptose was estimated by the sulphuric acid cysteine reaction of *Dische* (2). The optical density was examined in the range of 450 to 600 nm. A peak or a shoulder at 500 to 510 nm was taken as a qualitative indication of heptose. LPS from *Veillonella* Ve 5 (8) and *Salmonella typhi* 0901 were used as positive controls for KDO and heptose, respectively.

RESULTS

All LPS produced an absorption peak at 500–505 nm in the sulphuric acid cysteine reaction of *Dische*. Likewise, LPS from all strains hydrolysed with 0 02 N H_2SO_4 gave a positive reaction for KDO. The peak at 550 nm was low compared to that produced by similar amounts of *Veillonella* LPS (Fig 1). The thiobarbituric acid reaction was also performed on 1 mg samples of LPS from strain Fev 1 hydrolysed with 0 1 N HCl for ½ 1 4 and 18 h at 100° C. The peak height at 550 nm of the samples hydrolysed for ½ 1 and 4 h was the same. Samples hydrolysed for 18 h gave a low and broad plateau in the region of 530 to 550 nm.

Paper chromatography revealed the presence in LPS of all strains of glucosamine, glucose and heptose, identified as *glycero manno*-heptose. KDO was detected in trace amounts in all but a few LPS. Galactose or rhamnose was found in LPS from a few strains.

By GLC L-*glycero*-D-*manno*-heptose was found in LPS from all strains. In addition, most LPS gave another peak in the heptose region. By comparison with the chromatogram of LPS of *Salmonella typhimurium* SL 3150 (15), the sugar was identified as D-*glycero*-D-*manno*-heptose. A few LPS contai

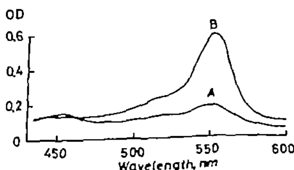


Fig 1 The absorption spectra in the thiobarbituric acid reaction of 2 mg of LPS from *F. nucleatum* strain F 13 (A) and *Veillonella* strain Ve 5 (B)

ned trace amounts of galactosamine, mannose or ribose (less than 0.1 per cent); otherwise the results of the GLC corresponded to those of paper chromatography.

Based on the results of the chromatographic and colorimetric examinations the LPS from the 20 *F. nucleatum* strains could be divided into six chemotypes (Table 1). The content of the different sugars, particularly glucose, varied from one LPS to another also within the same chemotype (Table 2).

TABLE 1 *Chemotypes of LPS Isolated from 20 Strains of* F. *nucleatum*

Chemotype	Origin of LPS	Sugar constituents						
		GlcN	Glc	KDO	L-hep	D-hep	Gal	Rha
I	VPI 4355 F19	× ×	× ×	× ×	× ×			
II	Fev 1 F5 F7 F8 F15 F10 F13, F14 F16	× ×	× ×	× ×	× ×	×		
III	ATCC 10953	× ×	× ×	× ×	× ×		×	
IV	F6 F11	× ×	× ×	× ×	× ×	×	×	
V	F1 F3 F9 F12, F17	× ×	× ×	× ×	× ×	×		×
VI	Fus-MC8	× ×	× ×	× ×	× ×			×

GlcN = glucosamine, Glc = glucose, KDO = 2-keto-3-deoxy-octonate, L-hep = L-glycero-D-manno-heptose, D-hep D-glycero-D-manno-heptose, Gal = galactose, Rha = rhamnose

TABLE 2 *Molar Ratios of Neutral Sugars Present in LPS Chemotypes I to VI, of* F. *nucleatum*

Chemotype	No of LPS	Molar ratios of				
		L-hep	D-hep	Glc	Gal	Rha
I	2	1		0 4 6 5		
II	9	1	0 3–0 9	0.4–2 2		
III	1	1		1	0 7	
IV	2	1	0 4 0 7	1 6–1 8	0 5 0 5	
V	5	1	0 2–0 8	0 9–4 4		0 2–2
VI	1	1		1 8		0 5

see legend to Table 1

DISCUSSION

The main implication of the present study is that *F. nucleatum* can be classified into chemotypes on the basis of the sugar composition of the cell wall lipopolysaccharide. It remains to be shown that there is a correlation between chemotypes and serotypes which undoubtedly exist in this organism (14).

With the exception of D-*glycero*-D-*manno*-heptose, the sugars found in the *F. nucleatum* LPS are common constituents of LPS from other bacterial species. D-*glycero*-D-*manno*-heptose, which may be an intermediate in the formation of L-*glycero*-D-*manno*-heptose (15), is present regularly in LPS of *Proteus vulgaris* (20). It is a common constituent of *P. mirabilis* LPS (11) and has been found in LPS isolated from *Yersinia pestis* (4) and the taxonomi-

(29) at 20° C for 15 min, and the LPS purified from the water phase by ultracentrifugation (100 000 × g for 90 min) and treatment with ribonuclease and deoxyribonuclease (7).

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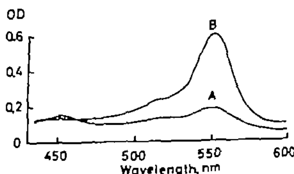


Fig 1 The absorption spectra in the thiobarbituric acid reaction of 2 mg of LPS from *F. nucleatum* strain F 13 (A) and *Veillonella* strain Ve 5 (B)

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cally remote organisms *Rhodopseudomonas gelatinosa* (27), *Rhodospirillum tenue* (26) and *Chromatium vinosum* (10).

The study confirmed the previous observations of apparently small amounts of KDO in *Fusobacterium* LPS (6 13) A low content of KDO was found also in LPS from *C vinosum* (10) Comparatively strong acid was required to release the thiobarbituric acid positive material from this LPS This does not seem to be true for *F nucleatum* LPS The finding of a low content of KDO relative to other sugar constituents, for instance L-*glycero*-D-*manno*-heptose, suggests that the linkage of the polysaccharide part of the *F nucleatum* LPS to the lipid A moiety may differ from that of enterobacterial LPS. However by mild acid hydrolysis, KDO may be converted to anhydro derivatives that do not react with the thiobarbituric acid reagent (24) Also KDO may be substituted in such a way that formylpyruvic acid, which is necessary for a positive thiobarturic acid reaction, is not produced on reaction with periodate (3).

The varying relative amounts of the sugar constituents (cf Table 2) suggest differences in structure of the LPS also within the same chemotype. It cannot be excluded, however that some of the glucose may have originated from small amounts of contaminating glucans In the previous study (13), the LPS of ATCC 10953 were found to lack glucose. It is probable that in that study the glucose band on the paper chromatograms was obscured by the stronger band given by heptose.

The quantitative GLC analysis revealed the presence in all LPS of glucosamine, L-*glycero*-D-*manno*-heptose and glucose. If glucosamine is present only in the lipid A, the findings may indicate a common polysaccharide core (or polysaccharide core backbone) structure involving KDO L-*glycero*-D-*manno*-heptose and glucose. Degradation studies are needed to answer this question Another unsolved problem is whether all O-antigenic specificities are carried by a number of repeating side chains linked to a single common polysaccharide core unit.

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cally remote organisms *Rhodopseudomonas gelatinosa* (27) *Rhodospirillum tenue* (26) and *Chromatium vinosum* (10)

The study confirmed the previous observations of apparently small amounts of KDO in *Fusobacterium* LPS (6 13). A low content of KDO was found also in LPS from *C vinosum* (10) Comparatively strong acid was required to release the thiobarbituric acid positive material from this LPS This does not seem to be true for *F nucleatum* LPS The finding of a low content of KDO relative to other sugar constituents for instance L-*glycero*-D-*manno*-heptose, suggests that the linkage of the polysaccharide part of the *F nucleatum* LPS to the lipid A moiety may differ from that of enterobacterial LPS. However by mild acid hydrolysis, KDO may be converted to anhydro derivatives that do not react with the thiobarbituric acid reagent (24) Also KDO may be substituted in such a way that formylpyruvic acid which is necessary for a positive thiobarturic acid reaction, is not produced on reaction with periodate (3)

The varying relative amounts of the sugar constituents (cf Table 2) suggest differences in structure of the LPS also within the same chemotype. It cannot be excluded however that some of the glucose may have originated from small amounts of contaminating glucans. In the previous study (13) the LPS of ATCC 10953 were found to lack glucose. It is probable that in that study the glucose band on the paper chromatograms was obscured by the stronger band given by heptose.

The quantitative GLC analysis revealed the presence in all LPS of glucosamine, L-*glycero*-D-*manno*-heptose and glucose. If glucosamine is present only in the lipid A the findings may indicate a common polysaccharide core (or polysaccharide core backbone) structure involving KDO L *glycero*-D-*manno*-heptose and glucose. Degradation studies are needed to answer this question. Another unsolved problem is whether all O-antigenic specificities are carried by a number of repeating side chains linked to a single common polysaccharide core unit.

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CHEMOTYPES OF *VEILLONELLA* LIPOPOLYSACCHARIDES

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Hofstad, T Chemotypes of *Veillonella* lipopolysaccharides Acta path microbiol scand Sect B, 86 47–50, 1978

Lipopolysaccharides (LPS) were isolated by phenol-water extraction from 34 strains of *Veillonella*, and examined by paper chromatography and colorimetric methods for the presence of neutral sugars, amino sugars and 2-keto-3-deoxy-octonate (KDO) Several preparations were also examined for neutral sugars by gas liquid chromatography The LPS had in common glucosamine, galactosamine, L-glycero-D-manno-heptose, glucose and KDO Most LPS contained galactose, and a few rhamnose D-glycero-D-manno-heptose was found in LPS from one of the strains Based on the sugar composition of the LPS, the *Veillonella* strains could be classified into four chemotypes

Key words *Veillonella* lipopolysaccharides, chemotypes

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Lipopolysaccharides (LPS) of *Veillonella* are potent endotoxins (20 21) and exhibit O-antigenic specificity (9 16) which depends on the polysaccharide component of the LPS. This component has been studied in some detail (6 8) LPS isolated from four oral *Veillonella* strains (Ve 5 Ve 6 Ve 8 and Ve 9) had in common 2-keto-3-deoxy-octonate (KDO), heptose, glucose, glucosamine and galactosamine, but varied with respect to the presence of galactose and ribose (8) KDO heptose and hexosamine were also present in LPS of the *Veillonella* type strains ATCC 10790, ATCC 17744 ATCC 17745 and ATCC 17748 (6) Glucose was present in LPS of all type strains, except ATCC 17745 LPS from ATCC 10790 and ATCC 17745 contained galactose, and LPS from ATCC 17744 contained rhamnose

The investigations indicated the presence in *Veillonella* LPS of chemotypes which might be partly responsible for the O-antigenic specificity Unusual findings were the presence in LPS from one of the oral *Veillonella* strains (Ve 5) of ribose, and especially the lack of glucose in the LPS of ATCC 17745 This sugar is only exceptionally absent (3) in LPS containing neutral sugars other than heptose.

We have, therefore, made new LPS preparations from the *Veillonella* type strains and the oral strains examined previously These and preparations of LPS from 26 other oral strains of *Veillonella* have been examined for sugar components by paper chromatography of acid hydrolysates and colorimetry and, to some extent, by gas liquid chromatography

MATERIALS AND METHODS

Strains

The origin of the *Veillonella* strains Ve 5 Ve 6 Ve 8 and Ve 9 has been reported previously (8) The additional 26 oral strains (cf Table 1) were isolated from the saliva of the same number of healthy human adults The type strains ATCC 10790 (*V parvula ss parvula*), ATCC 17744 (*V parvula ss atypica*), ATCC 17745 (*V alcalescens ss alcalescens*) and ATCC 17748 (*V alcalescens ss dispar*) were received from Dr K W Knox, Institute of Dental Research, United Dental Hospital, Sydney Australia

Cultural Conditions

Bulk cultivation was carried out as described earlier (8) The medium used was based on Trypticase and yeast extract and contained sodium lactate as energy source

present in small amounts. Most LPS contained glucose and a few rhamnose. Some preparations contained trace amounts of ribose. If the LPS contained galactose, this sugar and in a few LPS also galactosamine, were predominantly present in the hydrolysates made with 0.1 N H_2SO_4. Galactosamine or glucose was detected in the 0.1 N H_2SO_4 hydrolysates of the LPS which contained no galactose. By GLC, the heptose was identified as L-glycero-D-manno-heptose. In addition, LPS from one of the strains examined by GLC contained D-glycero-D-manno-heptose. Ribose was also detected in some LPS by GLC, but the amount varied considerably from one preparation of LPS to another from the same strain.

Based on the results of the colorimetric and chromatographic examinations, the LPS from the 34 strains of *Veillonella* could be divided into four chemotypes (Table 1). Table 2 shows that the relative amounts of the different neutral sugars of the LPS examined by GLC varied from one LPS to another within the same chemotype.

DISCUSSION

Analogous to *Salmonella* and other genera within *Enterobacteriaceae*, *Veillonella* can be classified into chemotypes on the basis of the sugar composition of the cell wall lipopolysaccharide. The majority of the LPS examined fell into chemotype II, characterized by the presence of galactose in addition to the common sugar constituents KDO, L-glycero-D-manno-heptose, glucose, glucosamine and galactosamine. The same sugars have been found in LPS isolated from serotypes B and X of *Neisseria meningitidis* (11). The sugar pattern characteristic of chemotype III has been reported in *N. perflava* (2). The sugar components of *Veillonella* chemotype I LPS are probably present in *N. sicca* (1, 10). Glycero-manno-heptose, glucose, galactose and glucosamine were found in LPS isolated from *N. gonorrhoeae* (15). Thus the *Veillonella* LPS have a sugar composition similar to that of the LPS of *Neisseriaceae*.

LPS from strain Ve 8, classified here as chemotype IV LPS, may perhaps represent a mutant LPS analogous to that of the rfa-657 strains of *Salmonella typhimurium* (14). However, D-glycero-D-manno-heptose is a frequent constituent of LPS from *Proteus* (13, 19), *Yersinia* (5), and *Fusobacterium* (4).

The results indicate that glucose, L-glycero-D-manno-heptose and KDO in *Veillonella* LPS constitute the polysaccharide core structure or its inner part. The release by mild acid hydrolysis of galactose or galactosamine in chemotype II LPS and of galactosamine or glucose in the other LPS chemotypes, suggests that these sugars are partly constituents of side chains. With respect to chemotype III LPS, which contains rhamnose, it is of relevance that *Johnson et al.* (12) have reported the presence in *Neisseria* LPS of three different oligosaccharides, one of which includes this sugar.

Numerous LPS serotypes exist in *Veillonella* (16), also within the same chemotype (*Ulofstad*, unpublished observations). This suggests a great variation in structural arrangement of the relatively few sugar components of the *Veillonella* LPS.

Previously we reported the presence of ribose in LPS from strain Ve 5 (8). In the present study variable amounts of ribose were found in the LPS-Ve 5 preparations examined, but ribose was not demonstrated in the supernatant fluid after mild acid degradation of the LPS (preliminary experiments). It is probable, therefore, that the occurrence of ribose in some of the LPS preparations was due to contamination with nucleic acid. The observation of *Hewett et al.* (6) that LPS isolated from ATCC 17745 lacked glucose was not substantiated by the present study.

This study was supported by the *Norwegian Research Council for Science and the Humanities*, and by *NIH Research Grant* No. DE 03821-01 from the United States Public Health Service.

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Isolation of LPS

Washed and acetone-dried whole cells were extracted with phenol water (23) for 30 min at room temperature (8) and purified from the water phase by ultracentrifugation (100 000 × g 60 min) and treatment with ribonuclease and deoxyribonuclease (7).

Paper Chromatography

Acid hydrolysis was performed in sealed tubes at 100° C with 3 N HCl for 3 h, with 6N HCl for 16 h, with 0 1 N H_2SO_4 for 10 min, and with N H_2SO_4 for 4 h. The samples were neutralized as described (8) and subjected to circular chromatography with n-butanol pyridine-water (6 4 3 by vol) Reducing sugars were detected with silver nitrate, aldoses with aniline hydrogen phthalate, and amino sugars with the Elson Morgan reagent (17) or ninhydrin.

Gas Liquid Chromatography (GLC)

Samples of LPS hydrolysed with 0 1 N HCl for 48 h at 100° C were neutralized with Amberlite IRA 410 HCO_3^- form, and the aldoses converted to alditol acetates, as described by *Sawardeker et al* (18) GLC was carried out in a Perkin Elmer 900 Gas Chromatograph with a flame ionization detector on a glass column (0 2 × 180 cm) packed with 3 per cent ECNSS-M on Gas-Chrom Q 100–120 mesh The flow of N_2 gas was 30 ml per min, and the column, injector and detector temperatures were 180° C or 190° C, 200 and 250° C, respectively D-xylose was used as internal standard

Colorimetric Determination of KDO

The thiobarbituric acid reaction (2?) was used on samples of LPS hydrolysed with 0 02 N H_2SO_4 for 20 min at 100° C The absorbance was examined in a Unicam SP 800 Ultraviolet Spectrophotometer A peak at 500–505 nm was recorded as a positive reaction

RESULTS

All LPS gave a high and distinct peak at 500–505 nm in the thiobarbituric acid reaction for KDO Paper chromatography of the different acid hydrolysates revealed the presence of heptose, glucose, glucosamine and galactosamine in LPS from all strains. In some LPS galactosamine seemed to be

TABLE 1 *Chemotypes of Veillonella LPS*

Chemotype	Origin of LPS	GlcN	GalN	Glc	KDO	L Hep	D-Hep	Gal	Rha
I	ATCC 17745 ATCC 17748								
	Ve 6 Ve 9 Ve 10 Ve 40	××	××	××	××	××			
II	ATCC 10790 Ve 5 Ve 7								
	Ve 11 Ve 20 Ve 22								
	Ve 26 Ve 30 Ve 42								
	Ve 46 Ve 47 Ve 48								
	Ve 50 Ve 51 Ve 53								
	Ve 54 Ve 60 Ve 61								
	Ve 62, Ve 63 Ve 67								
	Ve 68 Ve 70 Ve 328	××	××	××	××	××		×	
III	ATCC 17744 Ve 41								
	Ve 49	××	××	××	××	××			×
IV	Ve 8	××	××	××	××	××	×	×	

GlcN = glucosamine, GalN = galactosamine, Glc = glucose, KDO = 2 keto-3-deoxy-octonate, L Hep = L glycero-D-manno-heptose, D-Hep = D-glycero-D-manno-heptose, Gal = galactose, Rha = rhamnose

TABLE 2 *Molar Ratio of Neutral Sugars Present in LPS of 14 Strains of Veillonella*

Chemotype	No of LPS	Molar ratios of L Hep	D-Hep	Glc	Gal	Rha
I	3	1		1 5–4 1		
II	7	1		0 6–2 2	0 7–3 0	
III	2	1		2 6 3 2		0 7 0 9
IV	1	1	0 6	0 8	1 1	

Legend, see Table 1

present in small amounts. Most LPS contained galactose and a few rhamnose. Some preparations contained trace amounts of ribose. If the LPS contained galactose, this sugar and in a few LPS also galactosamine, were predominantly present in the hydrolysates made with 0.1 N H_2SO_4. Galactosamine or glucose was detected in the 0.1 N H_2SO_4 hydrolysates of the LPS which contained no galactose. By GLC, the heptose was identified as L-glycero-D-manno-heptose. In addition, LPS from one of the strains examined by GLC contained D-glycero-D-manno-heptose. Ribose was also detected in some LPS by GLC, but the amount varied considerably from one preparation of LPS to another from the same strain.

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All LPS gave a high and distinct peak at 500–50 nm in the thiobarbituric acid reaction for KDO Paper chromatography of the different acid hydrol sates revealed the presence of heptose, gluco glucosamine and galactosamine in LPS from a strains. In some LPS galactosamine seemed to b

TABLE 1 *Chemotypes of* Veillonella *LPS*

Chemotype	Origin of LPS	GlcN	GalN	Glc	KDO	L Hep	D-Hep	Gal	Rha
I	ATCC 17745 ATCC 17748 Ve 6 Ve 9 Ve 10 Ve 40	× ×	× ×	× ×	× ×	× ×			
II	ATCC 10790 Ve 5 Ve 7 Ve 11 Ve 20 Ve 22 Ve 26 Ve 30 Ve 42 Ve 46 Ve 47 Ve 48 Ve 50 Ve 51 Ve 53 Ve 54 Ve 60 Ve 61 Ve 62 Ve 63 Ve 67 Ve 68 Ve 70 Ve 328	× ×	× ×	× ×	× ×	× ×		×	
III	ATCC 17744 Ve 41 Ve 49	× ×	× ×	× ×	× ×	× ×			×
IV	Ve 8	× ×	× ×	× ×	× ×	× ×	×	×	

GlcN = glucosamine, GalN = galactosamine, Glc = glucose, KDO = 2-keto-3-deoxy-octonate, L Hep = L-*glycero*-D-*manno*-heptose, D-Hep = D-*glycero*-D-*manno*-heptose, Gal = galactose, Rha = rhamnose

TABLE 2 *Molar Ratio of Neutral Sugars Present in LPS of 14 Strains of* Veillonella

Chemotype	No of LPS	L Hep	D-Hep	Molar ratios of Glc	Gal	Rha
I	3	1		1 5–4 1		
II	7	1		0 6–2.2	0 7–3 0	
III	2	1		2 6 3 2		0 7 0 9
IV	1	1	0 6	0 8	1 1	

Legend, see Table 1

present in small amounts. Most LPS contained galactose and a few rhamnose. Some preparations contained trace amounts of ribose. If the LPS contained galactose, this sugar and in a few LPS also galactosamine, were predominantly present in the hydrolysates made with 0.1 N H_2SO_4. Galactosamine or glucose was detected in the 0.1 N H_2SO_4 hydrolysates of the LPS which contained no galactose. By GLC, the heptose was identified as L-glycero-D-manno-heptose. In addition, LPS from one of the strains examined by GLC contained D-glycero-D-manno-heptose. Ribose was also detected in some LPS by GLC but the amount varied considerably from one preparation of LPS to another from the same strain.

Based on the results of the colorimetric and chromatographic examinations, the LPS from the 34 strains of *Veillonella* could be divided into four chemotypes (Table 1). Table 2 shows that the relative amounts of the different neutral sugars of the LPS examined by GLC varied from one LPS to another within the same chemotype.

DISCUSSION

Analogous to *Salmonella* and other genera within *Enterobacteriaceae* *Veillonella* can be classified into chemotypes on the basis of the sugar composition of the cell wall lipopolysaccharide. The majority of the LPS examined fell into chemotype II characterized by the presence of galactose in addition to the common sugar constituents KDO, L-glycero-D-manno-heptose, glucose, glucosamine and galactosamine. The same sugars have been found in LPS isolated from serotypes B and Y of *Neisseria meningitidis* (11). The sugar pattern characteristic of chemotype III has been reported in *N. perflava* (2). The sugar components of *Veillonella* chemotype I LPS are probably present in *N. sicca* (1, 10). Glycero-manno-heptose, glucose, galactose and glucosamine were found in LPS isolated from *N. gonorrhoeae* (15). Thus the *Veillonella* LPS have a sugar composition similar to that of the LPS of *Neisseriaceae*.

LPS from strain Ve 8 classified here as chemotype IV LPS may perhaps represent a mutant LPS analogous to that of the *rfa*-657 strains of *Salmonella typhimurium* (14). However D-glycero-D-manno-heptose is a frequent constituent of LPS from *Proteus* (13, 19), *Yersinia* (5), and *Fusobacterium* (4).

The results indicate that glucose, L-glycero-D-manno-heptose and KDO in *Veillonella* LPS constitute the polysaccharide core structure or its inner part. The release by mild acid hydrolysis of galactose or galactosamine in chemotype II LPS and of galactosamine or glucose in the other LPS chemotypes, suggests that these sugars are partly constituents of side chains. With respect to chemotype III LPS, which contains rhamnose, it is of relevance that *Johnson et al.* (12) have reported the presence in *Neisseria* LPS of three different oligosaccharides, one of which includes this sugar.

Numerous LPS serotypes exist in *Veillonella* (16), also within the same chemotype (*Hofstad*, unpublished observations). This suggests a great variation in structural arrangement of the relatively few sugar components of the *Veillonella* LPS.

Previously we reported the presence of ribose in LPS from strain Ve 5 (8). In the present study variable amounts of ribose were found in the LPS-Ve 5 preparations examined, but ribose was not demonstrated in the supernatant fluid after mild acid degradation of the LPS (preliminary experiments). It is probable, therefore, that the occurrence of ribose in some of the LPS preparations was due to contamination with nucleic acid. The observation of *Hewett et al.* (6) that LPS isolated from ATCC 17745 lacked glucose was not substantiated by the present study.

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BRIEF REPORT

BOVINE PROTOTHECOSIS

A brief report of ten cases

Jessie Bodenhoff and P. Schmidt Madsen

Mycological Department, Statens Seruminstitut, Copenhagen, and Statens Veterinære Serumlaboratorium, Ringsted, Denmark

Bodenhoff, J. & Madsen, P. Schmidt. Bovine protothecosis. A brief report of ten cases. Acta path microbiol scand Sect. B 86 51–52, 1978

Prototheca zopfii was isolated repeatedly from milk samples from ten cows (of a herd of 192 dairy cows) with reduced milk yield and indurated mammary glands. The strain was moderately sensitive to streptomycin, polymyxin and gentamycin, but resistant or relatively resistant to other antibiotics and antimycotics commonly used in clinical practice.
An attempt to treat the infection with Ethidium bromide, which was found effective *in vitro*, did not succeed. The number of *Prototheca* excreted decreased, but a complete cure was not obtained.
In histological sections of the udder *Prototheca* cells were demonstrated both intracellularly and interstitially.

Key words. *Prototheca zopfii*, protothecosis, bovine, mastitis, bovine

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Accepted as submitted 26. 78

During routine examination of 192 dairy cows from one farm, microorganisms were isolated from milk samples from ten cows. At first the organisms were assumed to be atypical yeast cells, but a closer study revealed that they actually belonged to genus *Prototheca*.

Prototheca – first described by *Krüger* in 1894 – is a group of non-pigmented unicellular microorganisms belonging to the colourless algae.

Whereas numerous microorganisms among bacteria, fungi, protozoa, spirochaetes and viruses are well-known pathogens, algae are usually not associated with disease. Only in the last 20–25 years have they been mentioned at all in the medical literature, and then only rarely.

Geographically, *Prototheca* has been demonstrated in localities as far apart as Argentine, Germany, Puerto Rico, Sierra Leone, USA and Denmark.

Prototheca has been isolated from various sources such as slime flux of trees, lake and sea water, sewage, sludge, from skin scrapings and tissues from humans and animals, and from human sputum and faeces.

Protothecosis, the infection which may be produced by certain *Prototheca* species, was first described in a cow by *Lerche* in 1952. Lerche showed experimentally that the *Prototheca* strain concerned could give rise to mastitis, but histopathological studies were not performed.

Available evidence strongly suggests that the skin is the primary site of infection in both man and animals. The exact mechanism of infection is not known.

The most common form of protothecosis in man and animals seems to be the cutaneous variety which occurs either as a demarcated local skin lesion or as a generalized disease. Cutaneous manifestations were, in fact, described by *Davies et al.* who in 1964 were the first to prove that *Prototheca* can be pathogenic for man. They isolated a *Prototheca* strain from skin affections in an African rice farmer and also demonstrated its presence in the tissues.

Present material

During examination of a herd of 192 dairy cows, *Prototheca* was isolated repeatedly at weekly intervals from milk samples from ten of the cows. Clinically the cows were in good condition, though the affected quarters were indurated and the milk yield reduced.

For epidemiological reasons, it was attempted to trace the source of infection by inoculations from the milker

the fodder and the environment (including water holes and marlpits on the farm) but without result.

The actual strain grew well aerobically at 30–32°C on conventional culture media. In young cultures the colonies resembled colonies of yeast, but in older cultures on blood agar they were duller and drier. On Sabouraud agar they were finely shagreened, opalescent and transparent. The smell of the cultures on Sabouraud agar was unpleasant.

Prototheca cells are Gram-positive and catalase positive. In physiological studies of the isolated *Prototheca* strain by the method of Lodder 1970 no carbohydrates were fermented, but the strain assimilated glucose, glycerine, ethanol, n-propanol and lactate. Using Arnold and Ahearn's identification key (*Arnold & Ahearn* 1972) the strain in question was identified as *Prototheca zopfii*. Comparisons with type strains from the American Type Culture Collection have verified the diagnosis.

The cases of protothecosis reported in the literature were difficult to treat because of the relative resistance of *Prototheca* to the drugs usually applied against infections.

This observation is in good agreement with the results of sensitivity tests performed on the *Prototheca zopfii* strain under consideration here. The strain was moderately sensitive to streptomycin, polymyxin and gentamycin, but resistant or relatively resistant to other antibiotics and antimycotics commonly used in clinical practice.

As the antibiotic therapy applied to the affected cow proved ineffective, one of us (S. M.) thought that Ethidium bromide might prove effective in the treatment of the algal infection. Ethidium bromide – a phenanthridine derivative which can form a complex compound with DNA in cell nuclei – is used under the proprietary name of Homidium® in the treatment of trypanosome diseases in the tropics.

In serial dilutions on Sabouraud agar plates, the minimum inhibition concentration of Ethidium bromide was found to be 5 µg/ml.

However, mastitis was still present, even though treatment with Ethidium bromide of both the affected animals and an experimentally infected cow resulted in a fall in the excretion of *Prototheca* cells.

The experimentally infected and Ethidium bromide treated cow was killed and subjected to autopsy. *Prototheca* cells were demonstrated both intracellularly and interstitially in histological sections of the udder tissue.

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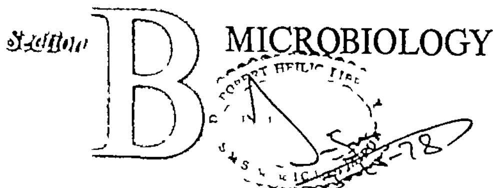
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THE ROLE OF CELL WALL CARBOHYDRATES IN BINDING OF MICROORGANISMS TO MOUSE PERITONEAL EXUDATE MACROPHAGES

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Freimer, N. B., Ögmundsdóttir, H. M., Blackwell, C. C., Sutherland, I. W., Graham, L. & Weir, D. M. The role of cell wall carbohydrates in binding of microorganisms to mouse peritoneal exudate macrophages. Acta path. microbiol. scand. Sect. B, 86: 53–57, 1978.

The recognition by macrophages of unopsonized bacteria was studied, employing a binding assay performed at 4° C. Various Gram positive and Gram negative bacteria were shown to bind to glass-adherent mouse peritoneal exudate cells under these conditions, *Str. pneumoniae* being the only exception. The binding could be inhibited by pretreatment of the macrophage monolayers with various monosaccharides. The role of particular components of the bacterial cell wall in binding was examined further using different strains of *K. aerogenes* and *S. typhimurium* with a known cell wall composition and mutant strains deficient in certain sugars. The ability of a particular constituent to inhibit binding was found to correlate closely with its presence in the bacterial cell wall. It is concluded, that this form of binding, mediated by cell wall carbohydrates represents a primitive recognition mechanism enabling phagocytes to bind microorganisms.

Key words: Macrophages; recognition; bacterial cell wall carbohydrates; *Salmonellae*; microbial pathogenicity.

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Accepted as submitted 17.x.77

The ability of phagocytes to adhere to various surfaces, to microorganisms and other particulate material in the absence of antibody or complement is a well known phenomenon (see *Stossel* 1975). The mechanisms underlying cell surface interactions of this type are poorly understood and in a previous report we showed the importance of carbohydrate constituents of the cell wall of the anaerobic coryneform *Corynebacterium parvum* in binding to mouse peritoneal exudate macrophages (*Ögmundsdóttir & Weir* 1976). The plasma membrane receptors for the cell wall carbohydrates appear to be glycoprotein in nature and the binding is dependent on divalent cations (*Ögmundsdóttir et al.* 1977).

Macrophage recognition abilities are of considerable potential importance in the understanding of immune mechanisms in infective disease and macrophage target cell interaction with altered tissue cells. In this report we have studied the role of cell wall carbohydrates of a variety of Gram +ve and −ve microorganisms in binding to mouse macrophages using mainly organisms with defined cell wall composition.

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TABLE 2 *Binding Inhibition — Klebsiella aerogenes Type 2*

K. aerogenes ($1-2 \times 10^{10}$/ml)	Percent binding (S.E.)	Inhibitory Sugars				
		Galactose	Glucose	Mannose	Glucosamine	Arabinose
Wild type 5055	18.1 (0.6)	−	+	−	−	−
Non-capsulated Mutant M2	19.0 (0.6)	+	+	−	+	−
Core Mutant M10B	19.1 (0.5)	−	+	−	+	−

organisms can readily be scored. The concentration of organisms required to achieve this binding level was usually between 10^9 and 10^{10} organisms/ml (see Table 1). In control experiments the formalin killing procedure was shown to have no effect on the binding ability of the organisms. *Str. pneumoniae* was the only organism tried that did not bind to the PEMs.

Inhibition of Binding by Monosaccharides and other Bacterial Wall Constituents

Using the working concentration of organisms, binding inhibition assays were performed with two commonly occurring cell wall sugars glucose and galactose. A sugar was regarded as inhibitory if it reduced binding to 60% of control values without inhibitor. Table 1 shows that glucose at a concentra-

TABLE 3 *Percent Binding of* S. typhimurium *Strains to Mouse PEMs and the Ability of Various Sugars and Cell Wall Constituents to Inhibit Binding. The Structure of the LPS of* S. typhimurium, *Wild Type and Mutants, Is Shown in the Schematic Formula*

O SOMATIC REPEAT UNIT: [O Acetyl, Abe 1→3 Man, Rha, Glc 1→4 Gal]

OUTER CORE: GlcNac — Glc II — Gal I; Gal II — Glc I — Hep II — Hep I

INNER CORE: KDO — Lipid A — Ethanolamine

	O somatic repeat unit	Outer core		Inner core
Genotype		rfaK		rfaE
Chemotype	smooth	Rb	Rc	Re
STRAIN	SL 1543	SL 1004	SL 1090	SL 1102
INHIBITION BY				
GLUCOSE				
GALACTOSE				
GLUCOSAMINE				
RHAMNOSE				
MANNOSE				
KDO	ND	ND	ND	
LIPID A				
ARABINOSE				

inhibition, − no inhibition (see text)

Abbreviations: Glc = glucose, Gal = galactose, Abe = abequose, GlcNac = N-acetyl glucosamine, Man = mannose, Rha = rhamnose, Hep = L-glycero-D-mannoheptose, KDO = 2-keto-3-deoxyoctonate, ND = not done.

Concentration of organisms $1-8 \times 10^9$/ml

MATERIALS AND METHODS

Animals. C3H mice (SPF) age 5 to 10 weeks were from the departmental breeding colony

Organisms. Staphylococcus albus, Staphylococcus aureus, Streptococcus viridans, Streptococcus pyogenes, Escherichia coli and *Bacillus anthracoides* were obtained from the departmental teaching collection. *Pseudomonas aeruginosa* NCTC 578 both mucoid and non-mucoid strains, were kindly provided by Dr *John Govan. Klebsiella aerogenes* serotype 2, NCTC 5055 a non capsulated mutant M2 and a rough mutant M10B as well as *Salmonella typhimurium* SL 1542 with a complete lipopolysaccharide (LPS) and rough mutants having incomplete LPS SL 1096 (*RfaK* chemotype Rb), SL 1099 (chemotype Rc) and SL 1102 (*rfaE* chemotype Re) were from the strain collection of Dr *Ian Sutherland* The organisms were grown in horse digest broth or nutrient broth harvested in log phase and killed by 24 h exposure to 0 5 % formalin at 4° C When living organisms were used they were stored at 4 C in 0 9% saline. Counting was performed in a Neubauer chamber 0 01 mm depth

Collection of peritoneal exudate cells (PE cells). 2 5 ml of Dulbecco s phosphate buffered saline (PBS) containing 10 IU/ml heparin, were injected i.p into C3H mice and after light massage withdrawn by means of a syringe The cell suspension, kept on ice, was centrifuged at 4 C at 250 × g for 7 minutes and, after removal of the supernatant, resuspended in Eagle s medium MEM (Wellcome Research Laboratories, Beckenham, England) without serum to give a final cell count of 2 to 4 × 10[6] ml

Preparation of monolayers Suspensions of PE cells were layered by pasteur pipette on to 7 × 20 mm "flying" glass coverslips in 1 2 × 7 4 cm glass tubes (WR tubes). Tubes were incubated at 37° C for 2 h and non-adherent cells removed by repeated washing with Dulbecco s PBS. Each coverslip then contained approximately 2 × 10[5] peritoneal exudate macrophages (PEMs)

Binding assay Coverslips with adherent PE cells were layered with a suspension of the appropriate organism at the concentrations shown in the tables in Dulbecco s PBS containing Ca^{++} and Mg^{++} ions (Dulbecco s PBS + B) and incubated for 2 hours at 4 C. Non-attached organisms were washed away with Dulbecco s PBS and the coverslips air dried, heat fixed, and stained by the Gram method. Microscopic examination of the monolayers was performed and PEMs with two or more attached organisms were counted. A total of 200 cells were counted on each slide, and the results expressed as the percentage of cells binding organisms.

Inhibition studies PEM cell monolayers were overlaid with solutions of various carbohydrates (hexoses, glucosamine, arabinose and 2 keto-3-deoxyoctonate (KDO), Sigma) and lipid A in Dulbecco s PBS + B Lipid A was prepared from freeze dried *K aerogenes* by phenol extraction (*Paxton & Sutherland* 1976) The concentration of inhibitors used was 10mM as preliminary experiments had shown that this was the minimum inhibitory concentration for glucose and galactose After fifteen minutes exposure to these materials at 4 C the monolayers were gently washed and the binding assay performed as described above Bacterial concentrations for the inhibition study were those for which 15–30 % binding had been observed in binding assays.

RESULTS

Binding of Various Gram Positive and Gram Negative Bacteria to PEMs

Binding assays were performed as described in Materials and Methods with a variety of Gram + ve and Gram −ve organisms. A working concentration of organisms was first determined that gave between 20 and 30% binding At this level of binding the PE cell monolayers with attached

TABLE 1 *Percent Binding of Organisms to Mouse PE Cell Monolayers and Inhibition by Glucose or Galactose*

Organism	Numbers/ml	Percent binding	S E	Inhibitory sugars	
				Glucose	Galactose
Str viridans	5 × 10[9]	33 7	1 0	+	−
Str pyogenes	5 × 10[10]	19 5	0 8	+	−
Str pneumoniae	up to 10[11]	0			
B anthracoides	3 × 10[9]	23 0	0 8	−	−
St albus	7 × 10[9]	21 1	1 0	+	+
St aureus	2 × 10[9]	19 5	0 9	+	+
E coli	7 × 10[8]	21 0	1 0	+	+
P aeruginosa (mucoid)	2 × 10[10]	19 9	1 0	+	+
P aeruginosa (non-mucoid)	1 × 10[10]	20 9	1 0	+	+
C parvum (10390)	3 × 10[10]	28 9	1 9	+	+

Data from *Ögmundsdóttir & Weir* 1976

TABLE 2. *Binding Inhibition – Klebsiella aerogenes Type 2*

K. aerogenes $(1-2 \times 10^{10}/ml)$	Percent binding (S.E.)	Inhibitory Sugars				
		Galactose	Glucose	Mannose	Glucosamine	Arabinose
Wild type 5055	18.1 (0.6)	–	+	–	–	–
Non-capsulated Mutant M2	19.0 (0.6)	+	+	–	+	–
Core Mutant M10B	19.1 (0.5)	–	+	–	+	–

organisms can readily be scored. The concentration of organisms required to achieve this binding level was usually between 10^9 and 10^{10} organisms/ml (see Table 1). In control experiments the formalin killing procedure was shown to have no effect on the binding ability of the organisms. *Str. pneumoniae* was the only organism tried that did not bind to the PEMs.

Inhibition of Binding by Monosaccharides and other Bacterial Wall Constituents

Using the working concentration of organisms, binding inhibition assays were performed with two commonly occurring cell wall sugars glucose and galactose. A sugar was regarded as inhibitory if it reduced binding to 60% of control values without inhibitor. Table 1 shows that glucose at a concentra-

TABLE 3. *Percent Binding of S. typhimurium Strains to Mouse PEMs and the Ability of Various Sugars and Cell Wall Constituents to Inhibit Binding. The Structure of the LPS of S. typhimurium, Wild Type and Mutants, Is Shown in the Schematic Formula*

	O SOMATIC REPEAT UNIT	OUTER CORE		INNER CORE
	[O Acetyl Abe 1→3 Man Rha Gal; Glc 1→4 Gal]	GlcNac / Glc II Gal I	Gal II / Glc I Hep II Hep I	KDO / Lipid A / Ethanolamine
Genotype / Chemotype	smooth	rfaK / Rb	Rc	rfaE / Re
STRAIN	SL 1542	SL 1004	SL 1000	SL 1102
INHIBITION BY				
GLUCOSE				
GALACTOSE				
GLUCOSAMINE				
RHAMNOSE				
MANNOSE				
KDO	ND			
LIPID A		ND	ND	
ARABINOSE				

+ inhibition, – no inhibition (see text)

Abbreviations: Glc = glucose, Gal = galactose, Abe = abequose, GlcNac = N-acetyl glucosamine, Man = mannose, Rha = rhamnose, Hep = L-glycero-D-mannoheptose, KDO = 2-keto-3-deoxyoctonate, ND = not done

Concentration of organisms $1-8 \times 10^9$/ml

tion of 10mM was capable of inhibiting binding of all organisms except *B anthracoides* in contrast galactose was ineffective with this and two additional organisms *Str viridans* and *Str pyogenes*

Similar binding inhibition assays were then performed with organisms of known cell wall constitution and with mutant strains with deficiencies of various sugars. Tables 2 and 3 show the results with 3 *K aerogenes* and 4 *S typhimurium* strains. The two *Klebsiella* mutants lacked capsule polysaccharides and M10B also had a defective lipopolysaccharide (LPS) core. Strain M10B contains only 0 2% (as % dry weight LPS) of galactose compared to between 20 and 30% in the wild type and mutant M2 (*Paxton & Sutherland* 1976). Table 2 shows that galactose has no inhibitory effect on the binding of the galactose deficient strain. Glucose which is present in a concentration of between 5 and 7% in all the strains is inhibitory with each of the organisms including the capsulated wild type. The bacterial cell wall also contains glucosamine which was inhibitory with the non-capsulated strain. *Heptose* and *KDO* are also present. The capsule contains both glucose and mannose but no galactose and mannose does not inhibit binding of any of the strains.

Table 3 shows the results of the inhibition assays with wild type *S typhimurium* (SL 1542) *and three mutants with deficiencies of sugars in the* 0 repeat unit and outer and inner core, as indicated in the table. The ability of sugars to inhibit binding *of a particular mutant is directly related to the* presence of the sugar with the exception of rhamnose and mannose. The rough mutant SL 1102 appears to bind by means of its KDO and lipid A components. Lipid A is a glycolipid containing substituted galactose. The degree of inhibition is usually down to 40–60% of controls, but lipid A reduces the binding of SL 1102 to 12–15%

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The results indicate that microorganisms bind to adherent mouse PE cells by means of cell wall and capsular sugars. The binding sites on the plasma membrane of the PE cells appear to have specificity for a variety of cell wall sugars in that a mutant strain of *K aerogenes* lacking in a cell wall sugar known to be involved in binding (galactose) still binds, presumably by means of its cell wall glucose component. *The presence of a binding site for* galactose is indicated by the ability of this sugar to partially inhibit the degree of binding of the *K aerogenes mutant known to contain galactose* as a cell wall component (Table 2) The importance of these two sugars in the binding of a variety of Gram +ve and Gram−ve organisms is indicated in Table 1

The structure of the lipopolysaccharides of *Klebsiella (Enterobacter) aerogenes* is by no means as well defined as that of *Salmonella* species. The presence of large amounts of nonacetylated galactose in the wild type and non-capsulated mutant (*Paxton & Sutherland* 1976) suggests that the 0 antigen is type 1 (*Björndal et al* 1971). (The Lipid A of some strains resembles that of *Salmonella* and justified its use for inhibiting the SL 1102 *Salmonella* mutant as no *Salmonella* Lipid A was available).

With the *S typhimurium* strains the schematic formula of which is shown in Table 3 the ability of sugars to inhibit binding is directly related to their presence in the 0 somatic repeat unit or the outer core. Mutants with missing sugars are not inhibited by those sugars. Mutant SL 1102 with only inner core components appears to bind by means of KDO and lipid A The ability of lipid A to inhibit the binding of the other strains as well as SL 1102 can possibly be explained by its galactose content. The only anomaly is the inhibition by rhamnose of SL 1096 that should not express the sugar This may be explained by the possibility that this mutant is "leaky" and synthesizing and expressing very small amounts of the wild type polysaccharide (*Lehmann et al* 1973)

The inability of mannose to inhibit binding of K aerogenes and the wild type *Salmonella* strain is noteworthy and a similar result was found in our *earlier studies with C parvum (Ögmundsdóttir & Weir* 1976). It is known that in the 0 repeat unit of the wild type *Salmonella* (SL 1542) the mannose is linked at a branch point to abequose and in *Klebsiella* wild type capsules mannose is linked in the same way to glucuronic acid The interaction with these sugars which are linked via hydrophilic and hydrophobic groups respectively may interfere with the expression of mannose as a potential binding sugar

These results extend our earlier observations on *C parvum* binding to mouse PE cells in which the importance of cell wall sugars in binding was clearly demonstrated (*Ögmundsdóttir & Weir 1976*) Work on the nature of the binding sites themselves indicates that they may be glycoprotein in nature (involving hydroxyl groups) *in view of their* susceptibility to proteolytic enzymes, β-galactosidase and periodate (*Ögmundsdóttir et al* 1977) The *requirement* for Ca and Mg in binding has also been noted and appears also to be a requirement for the binding of unopsonized albumin coated oil particles to phagocytes and for the binding of lymphocytes to macrophages (*Stossel 1975 Rosenthal et al* 1975).

In our view the binding of microorganisms to mouse PE cells, involving carbohydrates of the microbial cell wall and glycoprotein receptor sites on the macrophage plasma membrane, represents a primitive recognition mechanism enabling phagocytes to bind microorganisms prior to engulfment. It is likely that some organisms owe their pathogenicity to their ability to reduce the strength of binding by the presence of capsules that cover over sugars in the cell wall that would otherwise be involved in binding (and lead to phagocytosis). Other organisms may by releasing capsular material, block the phagocyte binding sites and thus allow the organism to escape the attention of the phagocytic cell. The capsular polysaccharide of *Cryptococcus neoformans* can be shown to inhibit the binding of this yeast to the phagocyte. Encapsulated organisms attached at low rates and were engulfed more slowly than non-encapsulated yeasts (*Kozel & Mastroianni* 1976). In our studies the presence of a capsule did not appear to alter the degree of binding with the *Klebsiella* strains although the presence of the capsule did prevent galactose in the cell wall from attaching to the galactose binding site. We have not studied the stages that occur after initial binding and cannot therefore comment on the effects on virulence of the organism.

In other situations where virulence depends on the ability of microorganisms to attach to and penetrate gut epithelium, as is the case with *Salmonella* strains, then binding is a requirement for pathogenicity. *Str. mutans* appears to adhere to the tooth surface by means of carbohydrates (glucans) attached via a glycosyl transferase to a surface polysaccharide containing galactose and glucose (*Gibbons & van Houte* 1971). It seems possible that the more sugars involved the greater the chance of attachment to, penetration and invasion of, host cells (particularly those cells with fewer binding sites). Table 3 confirms this suggestion in that the smooth virulent strain of *S. typhimurium* (SL 1542) binds to the phagocytic cells (and therefore presumably to other cells) by more sugars than *any of the* less virulent mutants. Although *S. typhimurium* wild type (LT2) appears to be initially more slowly phagocytosed than rough strains (*Nakano & Saito* 1968), phagocytosis and digestion is not a necessary sequel to binding (see *Smith* 1977). We suggest that the binding phenomenon described may have been the forerunner of the more effective binding mechanisms that involve cell bound opsonins and complement factors and would perhaps have provided a degree of protection from at least some potentially pathogenic microorganisms. Whether or not binding interaction by macrophages such as we have described extends to other forms of macrophage adherence remains to be elucidated. It is conceivable that interaction of this type may be responsible for macrophage recognition and attachment to a variety of carbohydrate containing surfaces such as transformed or altered tissue cells and carbohydrate antigenic determinants (*Weir & Ögmundsdóttir* 1977).

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Key words: *Escherichia coli*; *Staphylococcus aureus*; microcalorimetry; cephalexin; cephaloridin.

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The heat evolved by growing microorganisms can be registered by calorimetry. This technique can provide information regarding microbial growth in ecological systems such as water (10) and soil (2). Furthermore, calorimetry can be used for evaluation of optimal growth conditions in artificial culture systems (8).

We have recently used calorimetry as an approach for the study of the kinetics of the antibacterial action of antibiotics (3). By this technique, the capacity of related antibiotics, e.g. different tetracyclines, to depress heat production of cultures of *Escherichia coli* was found to differ markedly (6). Thus, differences in the time of onset of action of the drugs could be demonstrated. For each drug, there was a direct relationship between concentration and effect on heat production. On the other hand, when cultures of certain strains of *Staphylococcus aureus* and *E. coli* were exposed to increasing concentrations of some beta-lactam antibiotics, a "paradoxical" response was found, i.e.

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when the drugs were added in a certain range of concentrations, the effects on the heat production were less than when concentrations above or below that range were used (5).

In the present investigation the kinetics of the action of cephalexin and cephaloridin on *E coli* and *S aureus* were studied by means of ampoule and flow calorimetry The effects on heat production were related to the influence on viable counts, pH and optical density of the medium.

MATERIALS AND METHODS

Test Organisms and Culture Conditions

A strain of *E coli* (0 119 H:19) and an Oxford strain (483) of *S aureus* were tested. The bacteria used in the experiments had been stored and treated before use as described previously (6).

In the flow calorimetric studies, the bacteria were inoculated into Trypticase soy broth (TSB) (Oxoid), pH 7 0–7 1 and incubated at 37°C for 3 hours. They were then inoculated into the growth vessel of the calorimeter containing 600 ml of TSB to give a final concentration of 10^4 CFU ml^{-1} In the ampoule calorimetric experiments, the bacteria were subcultured from blood agar plates to TSB and incubated at 37°C for 4 hours before use. They were then inoculated into the antibiotic-containing culture medium of the test ampoule to give a final concentration of 10^4 CFU ml^{-1}

Antibiotics

Cephalexin and cephaloridin were supplied by Glaxo (Sweden) as a dry powder Stock solutions of the antibiotics (1 000 μg ml^{-1}) were prepared in phosphate buffered saline, pH 7 2. The final concentrations of the antibiotics (expressed in μg ml^{-1}) were based on the active portion of the drug.

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The MIC of the antibiotics used was determined by the broth dilution technique (12) using TSB. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of antibiotic that completely inhibited colony formation on blood agar plates In the MBC tests, aliquots of 0 05 ml of the broth cultures from the MIC tests, which contained increasing concentrations of antibiotic, were seeded onto blood agar plates after overnight incubation at 37°C

The MIC and MBC of cephalondin for the strain of *E coli* were 3 0 and 7 5 μg ml^{-1} and for cephalexin 4 5 and 7 5 μg ml^{-1} The MIC of cephalexin for the staphylococcal strain was 0 50 μg ml^{-1} while the MBC was 1 5 μg ml^{-1}

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The flow microcalorimeter used was a slightly modified version of the instrument described by *Monk & Wadso* (1) which is an instrument similar to the LKB (Stockholm Sweden) flow microcalorimeter model 10700-1 The thermo-sensible cell is a Teflon coil embedded in Wood s metal in a thin-walled aluminum box. The experimental procedures used in the calorimetric studies have been described in detail previously (6)

The growth vessel of the flow calorimeter was placed in a thermostatic bath holding 37 00°C. During the experimental period the broth was aerated constantly by bubbling sterile air through the growth medium. The medium was pumped through the calorimetric cell by means of a peristaltic pump (LKB model 10 200) at a flow rate of 28 ml h^{-1} The antibiotics were added to the broth cultures when there was a logarithmic increase in the heat effects developed. In each test, the addition was made at exactly the same heat production level i e 40 μW ml^{-1}

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Determination of Viable Count Optical Density and pH Studies of Bacterial Cell Morphology

Aliquots of the cultures were collected at chosen intervals and the number of colony forming units (CFU) ml^{-1} was determined by viable counts on blood agar plates. For the demonstration of cell-wall deficient bacterial forms, viable counts were also made on an L phase variant media consisting of heart infusion broth or agar (Difco) 45 ml supplemented by horse serum 20 ml yeast extract (bakers yeast 25% w/v) 5 ml, sucrose 8 5 g, $MgSO_4 \times 7H_2O$ 0 17 g, pH 7 2 The optical density at 540 nm (OD_{540}) of the broth samples was measured in a Linson 3 photometer (AB Ljungberg & Co Stockholm), and pH was measured by a pH meter (Radiometer Copenhagen) After gram-staining the bacteria were studied under a light microscope (× 1200)

Determination of the Concentration of Cephalexin

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Test for Beta-lactamase Production

The test strains of *E. coli* and *S. aureus* were studied for betalactamase production using chromogenic cephalosporin (compound 87/312, Glaxo Research Ltd, Greenford, England) (7). The same strains isolated at the end of calorimetric experiments with cephalexin were also tested.

RESULTS

Flow Calorimetric Experiments

E. coli. Fig. 1 shows the thermogram by a culture of *E. coli* to which cephalexin 9.0 μg ml^{-1} (i.e. 2 × MIC) was added in the logarithmic growth phase at a heat production of 40 μW ml^{-1}. There was an increase in the heat production and in the number of CFU ml^{-1} until the drug was added. Both parameters then decreased after which the heat production showed a transient increase before it rose definitely after approximately 11 hours. The number of CFU ml^{-1} had increased considerably 20 hours after the start of the experiment. At that time the pH, which had been stable up to 9 hours, had decreased, while the OD_{540}, which was low up to 9 hours, had increased.

The morphological studies showed that half-an-hour after the addition of cephalexin, the bacteria formed filamentous cells that sometimes appeared branched. These changes were successively more pronounced. Cell lysis was marked after approximately 9 hours. Examination after 21 hours showed spheroplast-like bacteria.

The MIC and MBC were 4.5 μg ml^{-1} and 7.5 μg ml^{-1} respectively at the start of the experiment. After 21 hours they had both increased to 200 μg ml^{-1}.

No beta-lactamase production of the test organisms could be demonstrated before or after exposure for 21 hours to cephalexin, 2 × MIC.

In another series of experiments, the effects of cephalexin (2 × MIC) on the heat production of the strain of *E. coli* were compared with those of cephaloridin (2 × MIC). Cephaloridin seemed to have more pronounced initial effects on the heat production than cephalexin (Fig. 2). On the other hand, the slater increase in heat came somewhat earlier for cephaloridin than for cephalexin.

S. aureus. Effects on heat production, viable count, pH, and OD_{540} similar to those found in *E.*

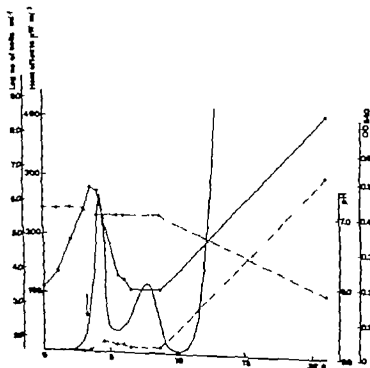


Fig. 1. Heat effects (——) produced by a strain of *Escherichia coli* exposed to 2 × MIC (9.0 μg ml^{-1}) of cephalexin. The antibiotics were added (indicated by arrow) in the logarithmic growth phase at a heat production of 40 μW ml^{-1}. Also shown are the number of colony-forming units ml^{-1} (●——●), optical density (OD_{540}) (●--●) and pH of the medium (● ●) at indicated time intervals. Flow calorimetric experiment.

when the drugs were added in a certain range of concentrations, the effects on the heat production were less than when concentrations above or below that range were used (5).

In the present investigation the kinetics of the action of cephalexin and cephaloridin on *E coli* and *S aureus* were studied by means of ampoule and flow calorimetry The effects on heat production were related to the influence on viable counts, pH and optical density of the medium

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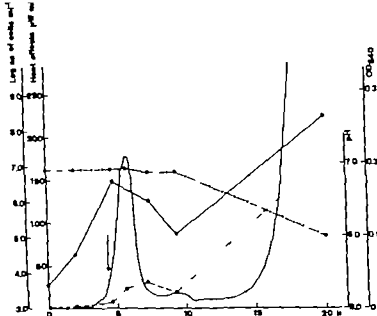


Fig. 3 Heat effects (——) produced by a strain of *Staphylococcus aureus* exposed to 5 × MIC (2.5 μg ml^{-1}) of cephalexin. The antibiotic was added (indicated by arrow) in the logarithmic growth phase at a heat production of 40 μW ml^{-1}. Also shown are the number of colony-forming units ml^{-1} (●——●), optical density (OD_{540}) (●--●) and pH of the medium (● ●) at indicated time intervals. Flow calorimetric experiment.

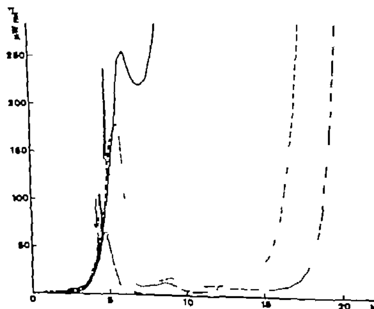


Fig. 4 Heat effects produced by a strain of *Staphylococcus aureus* exposed to cephalexin at increasing concentrations, i.e. 2 × MIC (1.0 μg ml^{-1}) (——), 5 × MIC (2.5 μg ml^{-1}) (——) 10 × MIC (5 μg ml^{-1}) ( ) and 100 × MIC (50 μg ml^{-1}) (-). The antibiotic was added (indicated by arrow) in the logarithmic growth phase at a heat production of 40 μW ml^{-1}. Flow calorimetric experiments.

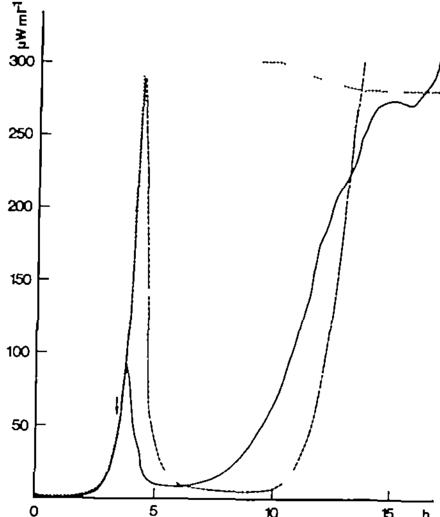


Fig 2 Heat effects produced by a strain of *Escherichia coli* cultured in the absence of antibiotics ( ) and when exposed to cephalexin in a concentration corresponding to 2 × MIC (9 0 μg ml⁻¹) (---) and cephaloridin 2 × MIC (6 0 μg ml⁻¹) (—). The antibiotics were added (indicated by arrow) in the logarithmic growth phase at a heat production of 40 μW ml⁻¹ Flow calorimetric experiments

coli were observed when cephalexin 2 5 μg ml⁻¹ (5 × MIC) was added to the culture of *S aureus* (Fig. 3) At a time when the heat effects and the viable counts had decreased, the morphological studies indicated that cell lysis had occurred. Fifteen hours after the start of the experiment, there was an increase in the heat production, which had been low for several hours. At 20 hours the number of CFU ml⁻¹ and OD_{540} had increased, whereas the pH had decreased as compared to the time at which the antibiotic was added The use of the L-phase variant medium gave the same number of CFU ml⁻¹ as found on the blood agar plates inoculated with samples collected at the same time. The bacterial cells isolated at this time showed normal morphology

The concentration of cephalexin remained constant during the experimental period

The MIC increased from 0 5 μg ml⁻¹ at the start of the experiment to 1 0 μg ml⁻¹ 20 hours later and the MBC increased from 1 5 μg ml⁻¹ to 16 0 μg ml⁻¹

No beta lactamase production of the test organisms could be demonstrated before or after exposure to cephalexin, 5 × MIC for 16 hours.

The effects of cephalexin on *S aureus* were dependent on the concentration of antibiotic used (Fig 4). An increase in the concentration of the drug from 2 × MIC to 100 × MIC depressed the heat production and caused a delay in the «later» development of heat. At a concentration of 100 × MIC, cephalexin abolished completely the late heat production during the experimental period

Ampoule Calorimetric Experiments

In ampoule calorimetric experiments, the strain of *E coli* was cultured in the presence of cephalexin and cephaloridin in concentrations corresponding to ½ and 1 × MIC (Fig 5). When using ½ × MIC the shapes of the thermograms were almost the

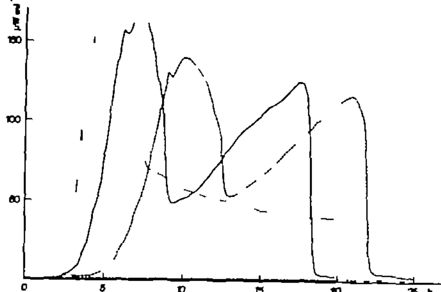


Fig 5 Heat effects produced by a strain of *Escherichia coli* cultured in the absence of antibiotics ( ), in the presence of cephalexin (——) in concentrations corresponding to ½ and 1 × MIC (2.3 and 4 6 μg ml⁻¹), *and in the presence of* cephaloridin (—) ½ and 1 × MIC (1 5 and 3 0 μg ml⁻¹). Drugs added at zero time. No heat effects were produced when concentrations corresponding to 1 × MIC were used. Ampoule calorimetric experiments.

same. In the experiment with cephaloridin the heat effects were recorded somewhat later than with cephalexin. When using 1 × MIC, no heat effects were produced during the experimental period.

DISCUSSION

It has been demonstrated previously that, in the calorimetric system used, at least 10^4–10^5 organisms ml⁻¹ are required before any heat effects can be registered (10) This observation is in accordance with the results of the present viable count determinations.

The heat registered was found to correspond only roughly to the number of CFU ml⁻¹ The number of organisms surviving and able to produce colonies after being transferred to fresh culture medium was determined by viable counts. However this technique does not provide information concerning the metabolic activity of the microorganisms Such information can be obtained by calorimetry which also gives a continuous recording of the metabolic activity of the organisms in the culture. To determine continuously the viable counts in a broth culture is however practically impossible Continuous registration of the optical density of a culture medium can be made, but this registration does not discriminate between viable and non viable organisms. Nor can information concerning the metabolic activity of the total cell mass be obtained by this technique. In the flow calorimetric experiments the addition of the antibiotics in the logarithmic growth phase was followed by a reduction of the number of CFU ml⁻¹ and in the heat effects evolved The second increase in the heat production was related in time to a rise in viable counts and OD_{540}

The second increase in heat production, which occurred after the introduction of the drugs, might have been explained by degradation or inactivation of the antibiotics, or by development of »tolerance« of the organism to the drugs (9) In our experiments with cephalexin these alternatives could be excluded No decrease in the concentration of the drug could be demonstrated during the experimental period There was no marked difference between MIC and MBC after the organisms had been exposed to the two cephalosporins, which is in contrast to findings with penicillin-tolerant organisms of *S aureus* (9) The results of the MIC determinations indicate a selection of resistant mutants as an explanation of the late heat increase. The morphological studies and the use of an L phase variant medium did not suggest that the late heat increase found when testing *S aureus* was due to cell wall-deficient bacteria resistant to the action of the cephalosporins tested.

When testing the strain of *S aureus* to increasing concentrations of cephalexin, the time period before the late heat increase occurred was directly correlated with the concentration of the antibiotic used. In an earlier microcalorimetric study (5), in which *S aureus* was exposed to increasing concentrations of benzylpenicillin and ampicillin we did not find any simple relation between concentration and response

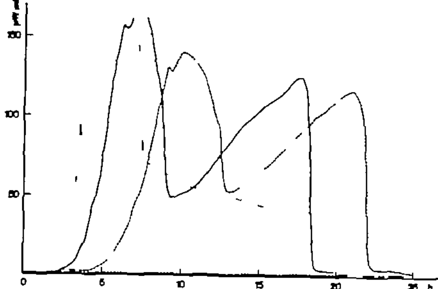


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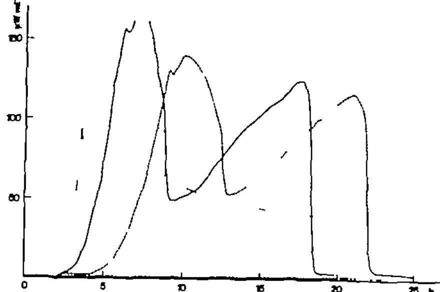


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Thus, the drugs in concentrations corresponding to 5 and 10 × MIC decreased the heat production less effectively than when concentrations corresponding to 2 and 100 × MIC were used. In this study a late increase in heat production was observed, except when very high concentrations of the drugs were used. Similar findings were obtained in studies of cinoxacin (4) and tetracyclines (6).

In the ampoule calorimetric experiments, the drug was present from the start, i.e. when the organisms were in the lag phase. No oxygen was added in these experiments as in the flow calorimetric experiments. The ampoule calorimetric technique has also other disadvantages as compared to flow calorimetry, e.g. it does not allow sampling during the experimental period. This contributes to the difficulty in interpretation of the thermograms obtained. The thermograms of the ampoule calorimetric experiments using cephalexin and cephaloridine indicated that the kinetics of the antibacterial activity of the two cephalosporins were similar. However, the heat production in the cephaloridine-containing culture occurred somewhat later than with cephalexin. This suggests that microcalorimetry can be used to study differences in, for instance, the time of onset of the antibacterial action of antibiotics.

The calorimetric experiments were performed in cooperation with the Thermochemistry Laboratory, Chemical Center, University of Lund. The study was supported by grant No 16X-4503 from the *Swedish Medical Research Council* and by a grant to the Thermochemistry Laboratory from the *Swedish National Board for Technical Development*. The kind support of Prof. *I. Wadsö* and the skilful help of Mrs *Eva Quarnström* and Miss *Ann-Catrine Petersson* are gratefully acknowledged.

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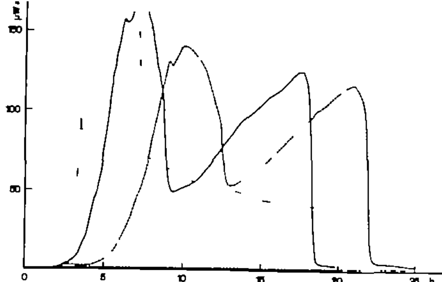


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It has been demonstrated previously that, in the calorimetric system used at least 10^4–10^5 organisms ml⁻¹ are required before any heat effects can be registered (10) This observation is in accordance with the results of the present viable count determinations.

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The second increase in heat production, which occurred after the introduction of the drugs, might have been explained by degradation or inactivation of the antibiotics, or by development of »tolerance« of the organism to the drugs (9). In our experiments with cephalexin, these alternatives could be excluded. No decrease in the concentration of the drug could be demonstrated during the experimental period There was no marked difference between MIC and MBC after the organisms had been exposed to the two cephalosporins, which is in contrast to findings with penicillin-tolerant organisms of *S aureus* (9) The results of the MIC determinations indicate a selection of resistant mutants as an explanation of the late heat increase The morphological studies and the use of an L phase variant medium did not suggest that the late heat increase found when testing *S aureus* was due to cell wall-deficient bacteria resistant to the action of the cephalosporins tested

When testing the strain of *S aureus* to increasing concentrations of cephalexin, the time period before the late heat increase occurred was directly correlated with the concentration of the antibiotic used In an earlier microcalorimetric study (5) in which *S aureus* was exposed to increasing concentrations of benzylpenicillin and ampicillin, we did not find any simple relation between concentration and response.

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BACITRACIN PRODUCTION BY THE HIGH YIELDING MUTANT *BACILLUS LICHENIFORMIS* STRAIN AL. STIMULATORY EFFECT OF L LEUCINE

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Haavik, H. I. & Vessia, Berit. Bacitracin production by the high-yielding mutant *Bacillus licheniformis* strain AL. stimulatory effect of L-leucine. Acta path. microbiol. scand. Sect. B, 86: 67–70, 1978.

The high-yielding mutant *Bacillus licheniformis* AL produced only small amounts of bacitracin in the chemically defined M2 medium. L-leucine markedly stimulated bacitracin production and restored the mutant strain to its place as a superior producer as compared to *Bacillus licheniformis* ATCC 10716. Leucine also stimulated the growth rate of the mutant. The stimulatory effect of leucine on bacitracin production is discussed in relation to control mechanisms and overproduction of antibiotics.

Key words: Bacitracin, high-yielding mutant, stimulation by leucine.

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Industrial production of the important antibiotics relies on certain high-producing mutants which are more or less remote descendants of the original antibiotic producing microorganisms once isolated from nature. The high-yielding mutants have been developed in various mutation and selection programmes (1,3).

Although these programmes have been time-consuming and laborious, we still have a poor knowledge of the cellular mechanisms responsible for the overproduction of antibiotics in these superior mutants (3,10).

However, some suggestions have been made. *Dulaney* (4) has suggested that blocking of alternate pathways of intermediates or blocking of further degradation of the antibiotic may increase yields. *Demain* (2,3) has put forward the interesting hypotheses that superior mutants may have altered control mechanisms for antibiotic synthesis.

We have studied a high-yielding bacitracin producing mutant, *Bacillus licheniformis* strain AL, and compared it with the original bacitracin producing culture *Bacillus licheniformis* ATCC 10716, in order to obtain some information regarding overproduction of this antibiotic.

MATERIALS AND METHODS

Reagents. All reagents used were of analytical grade, except the ingredients of the complex industrial production medium.

Organisms. The bacitracin producing strains *Bacillus licheniformis* AL and ATCC 10716 were kept as spore suspensions at 4° C.

Media and growth conditions. The industrial production medium consisted of a mixture of some flours and salts with soy bean flour as the main component. The chemically defined M2 medium consisted of glutamic acid and salts (9). The constituent amino acids of bacitracin (see Table 2) were added singly to the medium before autoclaving at 121° C for 20 min. The inoculum was 0.5 ml of an overnight culture in the M2 medium. Bacteria were grown in 500 ml Erlenmeyer flasks (containing 50 ml medium) in a 37° C water bath with

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shaking (120 rev/min) or on a New Brunswick rotatory shaker (260 rev/min).

Growth Bacterial growth was measured as extinction at 650 nm (E 650) in a Spectronic 20 spectrophotometer

Microbiological assay Bacitracin was determined by an agar diffusion method, as described previously (7).

RESULTS

In a complex production medium, the high-yielding mutant *Bacillus licheniformis* strain AL produced about 50 per cent more bacitracin than the original bacitracin producing strain *Bacillus licheniformis* ATCC 10716 Both strains seemed to produce bacitracin throughout the period of active growth.

TABLE 1 *Growth and Bacitracin Production by* B licheniformis *strain ATCC 10716 and AL in M2 Medium*

Strain	Doubling time (minutes)	Maximum growth (E 650)	Maximum bacitracin production (i.u /ml)
ATCC 10716	69	9–10	10–13
AL	100	9–10	1–2

In the chemically defined M2 medium, *B licheniformis* ATCC 10716 produced about 12 i.u / ml (Table 1) The high-yielding mutant *B licheniformis* AL grew well in the M2 medium and reached about the same maximum crop as *B licheniformis* ATCC 10716 However strain AL showed a longer lag period and a markedly slower growth rate than strain ATCC 10716 The mutant strain was a poor bacitracin producer in the M2 medium (Table 1). Very small amounts of bacitracin accumulated during growth, with maximum production towards end of growth of about 1.5 i.u./ ml.

TABLE 2. *Effect of the Constituent Amino Acids on Bacitracin Production by* B licheniformis *strain AL*

Amino acid added (1 g/1)	Growth (E650)	Bacitracin (i.u./ml)
none	8 8	1 5
L-isoleucine	8 8	0 7
L-cysteine	8 8	0 4
L leucine	9 3	22 4
D-glutamic acid	8 6	1 0
L lysine	9 1	2 0
D-ornithine	8 4	1 2
D-phenylalanine	8 8	3 6
L histidine	7 6	1 6
D-asparagine	5 4	0 6
L-aspartic acid	8 6	2 0
L-ornithine (a)	8 4	1 5
L-phenylalanine (a)	9 0	1 5
L-asparagine (a)	8 8	2.4

(a) L-isomers of the corresponding constituent D-amino acids (6)
Results after incubation for 18 hours

TABLE 3 *Effect of Leucine upon Bacitracin Production by* B licheniformis *strain ATCC 10716 and AL*

Added leucine (g/1)	Strain ATCC 10716 Growth (E650)	Strain ATCC 10716 Bacitracin (i.u./ml)	Strain AL Growth (E650)	Strain AL Bacitracin (i.u./ml)
none	8 4	11 8	9.5	1.3
0 2	9 5	11.2	9 7	12.7
0.5	8 8	10 2	9 2	20.8
0 7	8 7	11 6	9 4	22.4
1 0	9 0	13 0	8.8	21 6
2 0	9 1	12.4	10 0	20 8

Results after incubation for 18 hours.

However when the constituent amino acids of bacitracin were added to the M2 medium (singly), the bacitracin production of the mutant AL was markedly stimulated in the presence of L-leucine (Table 2). None of the other constituent amino acids showed this stimulatory effect. Nor did the L isomers of the constituent D-amino acids stimulate the bacitracin production of strain AL, (Table 2).

TABLE 4 *Growth and Bacitracin Production by* B licheniformis *strain ATCC 10716 and AL in M2 Medium with Leucine (0 7 g/1)*

Strain	Doubling time (minutes)	Maximum growth (E 650)	Maximum bacitracin production (i u./ml)
ATCC 10716	63	9–10	10–13
AL	79	9–10	20–22

Table 2 shows the results obtained after incubation for 18 hours. Results obtained at earlier or later stages of the growth phase give the same picture viz. a marked stimulatory effect of L-leucine. D-asparagine resulted in an inhibition of growth.

Maximum bacitracin production occurred with 0 7 g leucine/1 in the M2 medium A further increase of this amino acid had no stimulatory effect

(Table 3). L-leucine had no stimulatory effect upon bacitracin production by *B. licheniformis* ATCC 10716 (Table 3). The growth rate of the mutant strain AL was markedly increased in the presence of leucine (Table 4).

L-leucine did not stimulate the bacitracin production by strain AL in the complex production medium.

DISCUSSION

Mutation and selection have provided useful mutants with increased antibiotic production. However, we know very little about the cellular mechanisms responsible for the high antibiotic production in these mutants. One suggestion is that mutations which result in blocking of alternate pathways of intermediates involved in antibiotic biosynthesis may provide an increased flow of these intermediates towards antibiotic formation, thus increasing the yield (4). This hypothesis may explain why an increase in antibiotic production usually occurs in a stepwise manner and why improved mutants are increasingly difficult to obtain as yields are increased. The missing end products from the blocked pathways are supposed to be provided from the complex fermentation media employed.

The high yielding mutant *B. licheniformis* AL has been subject to several mutagenic treatments before its high productivity was expressed. This industrial production mutant can still grow in the chemically defined M2 medium. No vital pathways can therefore have been blocked in this mutant.

However, the mutant strain AL behaves differently from strain ATCC 10716 in the M2 medium. *B. licheniformis* AL shows a markedly slower growth rate and a poor bacitracin production in the M2 medium as compared to *B. licheniformis* ATCC 10716. It is interesting that a mutant which is a superior producer in one medium may be an inferior producer in another.

The amino acid L-leucine markedly stimulated the growth rate and the bacitracin production by the mutant strain in the M2 medium. The addition of 0.5 g leucine/l or more restored strain AL to its place as a superior producer of bacitracin in a defined medium. L-leucine had no stimulatory effect on bacitracin production by strain ATCC 10716, and there was only a slight stimulatory effect on the growth rate.

L-leucine was the only constituent amino acid of bacitracin which was able to restore the high yielding mutant AL to its place as a superior producer in the chemically defined medium. However, a slight stimulatory effect upon bacitracin production was observed in the presence of D-phenylalanine.

Snoke (14) reported that D-phenylalanine inhibited bacitracin production by *B. licheniformis* ATCC 10716 when no L-phenylalanine was added. He suggested that the D-isomer prevented the utilization of the L-isomer, that the L-isomers were used as such in the biosynthesis of bacitracin, and that conversion to the D-form does not occur with the free amino acid. Later *Pfaender et al.* (11) reported that none of the constituent D-amino acids of bacitracin (glutamic acid, ornithine, phenylalanine and asparagine) supported bacitracin production in a crude cell-free extract of *B. licheniformis* ATCC 10716. In contrast to this, *Frøyshov & Laland* (6) have shown that the D-isomers of glutamic acid, phenylalanine and asparagine could support bacitracin production in their cell-free preparation of *B. licheniformis* ATCC 10716. The stimulatory effect of D-phenylalanine on bacitracin production by strain AL is in line with the results obtained by *Frøyshov & Laland* (6).

The bacitracin synthetase multienzyme complexes of strain ATCC 10716 and strain AL have been compared in vitro (8). There were no differences between the two synthetases. The amino acids of the bacitracin molecule (including L-leucine) were activated in a similar manner and different enzyme components could be interchanged without disturbing bacitracin biosynthesis. This indicates that the two strains have similar bacitracin synthetases.

The most interesting hypothesis concerning high production of antibiotics is that the known control mechanisms in the metabolism, i.e. induction, feedback regulation, catabolite regulation and energy charge regulation, may also affect antibiotic biosynthesis, and that the high-yielding mutants selected from the various screening programmes may have defects or modifications in these control mechanisms (2,3).

It is reported that growth of *E. coli* in the presence of leucine results in a decrease of leucine transport activity (13). Furthermore, it has been observed that leucine activated as leucyl-tRNA is able to repress branched-chain amino acid transport (15). Thus both exogenous and endogenous leucine may participate in the control of leucine uptake.

Leucine is supposed to play a central role in metabolism by inhibiting or stimulating several important enzymes (16). *Fraser & Newman* (5) have suggested that intracellular leucine levels may serve as a signal for nitrogen scavenging during periods of amino acid imbalance. Thus leucine may be an alarmone analogous to cAMP and ppGpp. A more or less direct connection between internal leucine levels and high bacitracin production is therefore an interesting possibility.

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none	8 8	1 5
L-isoleucine	8 8	0 7
L-cysteine	8 8	0 4
L-leucine	9 3	22 4
D-glutamic acid	8 6	1 0
L-lysine	9 1	2.0
D-ornithine	8 4	1 2
D-phenylalanine	8 8	3 6
L-histidine	7 6	1 6
D-asparagine	5 4	0 6
L-aspartic acid	8 6	2 0
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none	8.4	11 8	9.5	1.3
0 2	9.5	11.2	9 7	12.
0.5	8 8	10 2	9.2	20 8
0 7	8 7	11 6	9 4	22.4
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Probably the mutant *B licheniformis* AL has a reduced capacity to synthesize leucine and thus has a less firm control of internal leucine levels. This may explain why the addition of leucine markedly increases the growth rate of the mutant.

It is also possible that low internal leucine may result in a more or less uncontrolled uptake of this amino acid, and the resulting high internal leucine levels may stimulate bacitracin production.

Although strain AL has need of leucine for rapid growth in the M2 medium, the mutant still produces small amounts of bacitracin in the absence of exogenous leucine. This indicates that small amounts of bacitracin may be necessary for the cells (8). It is also possible that the cells may have different utilization of exogenous and endogenous amino acid pools for antibiotic and protein synthesis as may be the case with actinomycin production by *Streptomyces antibioticus* (12).

This investigation supports the hypothesis that high-yielding antibiotic-producing mutants may have various defects or modifications in control mechanisms.

Grateful thanks are due to Mr *T Hayland* Director of Research and Development, for his support, and to Mr *S Thomassen* for commenting on the manuscript.

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DEOXYRIBONUCLEIC ACID HYBRIDIZATION BETWEEN DIFFERENT SPECIES OF MYCOBACTERIA

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Baess, I. & Bentzon, M. Weis. Deoxyribonucleic acid hybridization between different species of mycobacteria. Acta path. microbiol. scand. Sect. B, 86: 71–76, 1978.

The homology percentages between DNA from *M. smegmatis* ATCC 607 and DNA from nine various species of mycobacteria have been determined. DNA-DNA hybridization was measured in a spectrophotometer. The technique, calculation of results and the uncertainty of the method are described.

Key words: Nucleic acid hybridization; mycobacterium; classification; regression analysis.

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Throughout several decades numerous strains of mycobacteria, both pathogenic and non-pathogenic for man and animals, have been isolated and given names in many countries. Much work has been done in examining these bacteria and much effort has been made to coordinate the results of the various laboratories on an international basis, for instance, the results of biochemical tests. There are still many unclarified questions as regards the taxonomy of the mycobacteria, and divergent opinions and doubts still exist concerning the species status of a number of strains.

It can be assumed that DNA-DNA hybridization between different mycobacteria can provide definite solutions to these problems. A method has therefore been developed with this object in view. In order to avoid radioactive labelling of the DNA, we chose to measure the hybridization kinetics optically in a spectrophotometer. The method is a modification of *Bradley's* (1973) method, the principle of which originates from *Seidler & Mandel* (1971).

MATERIAL AND METHODS

Bacterial strains. The strains used for DNA isolation are listed in Table 1.

TABLE 1. *Strains Used for Isolation of DNA*

M. bovis, BCG	Copenhagen	
M. kansasii	ATCC	12478
M. marinum	ATCC	927
M. gordonae	ATCC	14470
M. avium	ATCC	25291
M. gastri	ATCC	25158
M. smegmatis	ATCC	607
M. smegmatis	No.	108
M. phlei	ATCC	27086
M. fortuitum	ATCC	9820

Isolation and purification of DNA. The method, the mechanical disruption of the bacteria, the slight modification of Marmur's method of isolation of the DNA and the purification of DNA with cetyltrimethylammonium bromide (CTAB) have been described previously (*Baess* 1974).

Probably the mutant *B licheniformis* AL has a reduced capacity to synthesize leucine and thus has a less fine control of internal leucine levels. This may explain why the addition of leucine markedly increases the growth rate of the mutant.

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Although strain AL has need of leucine for rapid growth in the M2 medium, the mutant still produces small amounts of bacitracin in the absence of exogenous leucine. This indicates that small amounts of bacitracin may be necessary for the cells (8). It is also possible that the cells may have different utilization of exogenous and endogenous amino acid pools for antibiotic and protein synthesis, as may be the case with actinomycin production by *Streptomyces antibioticus* (12).

This investigation supports the hypothesis that high yielding antibiotic-producing mutants may have various defects or modifications in control mechanisms.

Grateful thanks are due to Mr *T Heyland* Director of Research and Development, for his support, and to Mr *S Thomessen* for commenting on the manuscript.

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Acta path. microbiol. scand. Sect. B, 86: 71–76, 1978.

DEOXYRIBONUCLEIC ACID HYBRIDIZ
BETWEEN DIFFERENT
SPECIES OF MYCOBACTERIA

I. BAESS AND M. WEIS BENTZON

The Tuberculosis and Biostatistical Departments, Statens Seruminstitut, Copenhagen, D...

Baess, I. & Bentzon, M. Weis. Deoxyribonucleic acid hybridization between different speci... mycobacteria. Acta path. microbiol. scand. Sect. B, 86: 71–76, 1978.

The homology percentages between DNA from *M. smegmatis* ATCC 607 and DNA from some vari... species of mycobacteria have been determined. DNA/DNA hybridization was measured in ... spectrophotometer. The technique, calculation of results and the uncertainty of the method ... described.

Key words: Nucleic acid hybridization; mycobacterium; classification; regression analysis.

I. Baess, The Tuberculosis Department, Statens Seruminstitut, Amager Boulevard 80, DK 2... Copenhagen S, Denmark.

Accepted as submitted 21.x.77

Throughout several decades numerous strains of mycobacteria, both pathogenic and non-pathogenic for man and animals, have been isolated and given names in many countries. Much work has been done in examining these bacteria and much effort has been made to coordinate the results of the various laboratories on an international basis, for instance, the results of biochemical tests. There are still many unclarified questions as regards the taxonomy of the mycobacteria, and divergent opinions and doubts still exist concerning the species status of a number of strains.

It can be assumed that DNA-DNA hybridization between different mycobacteria can provide definite solutions to these problems. A method has therefore been developed with this object in view. In order to avoid radioactive labelling of the DNA, we chose to measure the hybridization kinetics optically in a spectrophotometer. The method is a modification of *Bradley's* (1973) method, the principle of which originates from *Seidler & Mandel* (1971).

MATERIAL AND METHODS

Bacterial strains. The strains used for DNA isolation are listed in Table I.

TABLE I. *Strains Used for Isolation of DNA*

M. bovis, BCG	Copenhagen	
M. kansasii	ATCC	12478
M. marinum	ATCC	927
M. gordonae	ATCC	14470
M. avium	ATCC	25291
M. gastri	ATCC	25158
M. smegmatis	ATCC	607
M. smegmatis	No	108
M. phlei	ATCC	27086
M. fortuitum	ATCC	9820

Isolation and purification of DNA. The method, the mechanical disruption of the bacteria, the slight modification of Marmur's method of isolation of the DNA and the purification of DNA with cetyltrimethylammonium bromide (CTAB) have been described previously (*Baess* 1974).

Probably the mutant *B lichenlformis* AL has a reduced capacity to synthesize leucine and thus has a less fine control of internal leucine levels. This may explain why the addition of leucine markedly increases the growth rate of the mutant.

It is also possible that low internal leucine may result in a more or less uncontrolled uptake of this amino acid, and the resulting high internal leucine levels may stimulate bacitracin production.

Although strain AL has need of leucine for rapid growth in the M2 medium, the mutant still produces small amounts of bacitracin in the absence of exogenous leucine. This indicates that small amounts of bacitracin may be necessary for the cells (8) It is also possible that the cells may have different utilization of exogenous and endogenous amino acid pools for antibiotic and protein synthesis, as may be the case with actinomycin production by *Streptomyces antibioticus* (12)

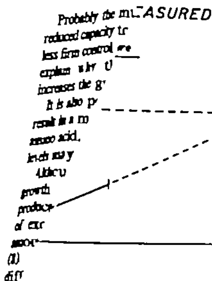
This investigation supports the hypothesis that high yielding antibiotic-producing mutants may have various defects or modifications in control mechanisms.

Grateful thanks are due to Mr *T Høvland* Director of Research and Development, for his support, and to Mr *S Thomassen* for commenting on the manuscript

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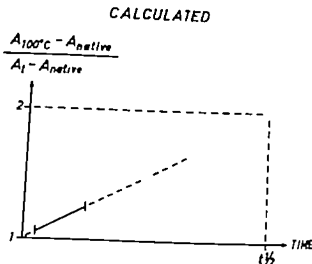


Fig. 1 Kinetics of the DNA DNA hybridization.
A Absorbance measured at 270 nm of DNA in native condition, at 100°C at time 0 and at time t.
—| Part of the experiment used for calculations.
Left side: Measured values.
Right side: Calculated values after extrapolation to A at time 0

Shearing of DNA The DNA was sheared at a concentration of 800 μg/ml in a French pressure cell press at 20 000 p.s.i. The sedimentation coefficient of the sheared DNA in 1 x SSC (0.15 M sodium chloride, 0.015 M trisodium citrate) was determined by analytical ultracentrifugation. From the extrapolated value

$$(S^{c=0}_{25\,w})$$

a molecular weight of 681 000 daltons (± 5 per cent) was calculated *(Crothers & Zimm 1965)*

Hybridization The reassociation of two bacterial DNAs as compared with the hybridization of the mixture of these, was measured optically in a spectrophotometer. The $C_0t\frac{1}{2}$ of a DNA is defined by *Britten & Kohne* (1966) as the concentration of DNA in moles of nucleotides per l multiplied by the time in seconds required for 50 per cent reassociation. If two bacterial strains have identical DNAs, the $C_0t\frac{1}{2}$ of the mixture of their DNAs is the same as the $C_0t\frac{1}{2}$ of each single DNA. If two bacterial strains have no mutual DNA sequences, the $C_0t\frac{1}{2}$ of the mixture will be the sum of their $C_0t\frac{1}{2}$s. This is described by *Seidler & Mandel* (1971) and expressed in their equation which is transformed by *Bradley* (1973) to homology percentage = 100 + 100 ($C_0t\frac{1}{2}$ A + $C_0t\frac{1}{2}$ B − 2 $C_0t\frac{1}{2}$ M)/ ($C_0t\frac{1}{2}$ A + $C_0t\frac{1}{2}$ B). A and B are the two bacteria to be compared and M the mixture

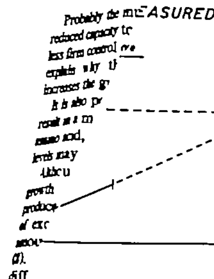
After some preliminary experiments with salt concentrations from 1 to 6 SSC, DNA concentrations of about 10, 20 and 40 μg/ml, and formamide concentrations of 25 and 50 per cent, we decided to perform the hybridizations with 40 μg DNA/ml in 3 SSC and 25 per cent formamide. Formamide is necessary in order to reduce the temperature in the experiment. The hybridization temperature, 25°C below T_m, in the mixture used was about 60°C. T_m was determined each time a new combination of DNAs was used, and in the preliminary experiments, for the various concentrations of SSC and formamide. The temperature in each experiment was controlled by thermistor in a cuvette placed between the reference and the three DNAs, A, B and M

After measurement of the concentration of the DNAs at 260 nm, the cuvettes were heated to between 99°C and 100°C and kept there for 15 minutes. The temperature was then changed as quickly as possible by changing to another oil bath adjusted beforehand to the hybridization temperature. When the temperature was reached, the absorbances were measured at 2 minute intervals. The absorbances in the hybridization experiments were measured at 270 nm because the influence of the formamide is less there than at 260 nm

Calculation of the $C_0t\frac{1}{2}$s and of the homology percentages between the DNA of two bacteria was made on the basis of the absorbances at room temperature and 100°C and the rate of absorbance decrease during hybridization. The second-order reaction plot of the experiment is shown in Fig. 1. The ordinate in the left hand part of the figure is

$$y_t = (A_{100°C} - A_{native})/(A_t - A_{native})$$

where A_t is the value read at time t. The time for the changeover to the oil bath with the hybridization temperature is chosen as t = 0. The curve is not correct, since $A_{100°C}$ is different from A_0, which corresponds to the absorbance of DNA at 25°C below T_m at time 0. This is due to the physical change in the DNAs as the temperature decreases. The slope of the line and the intercept at the y axis are determined by regression analysis *(Diem & Lentner 1971)*. These are designated b_y and a_{yt} respectively



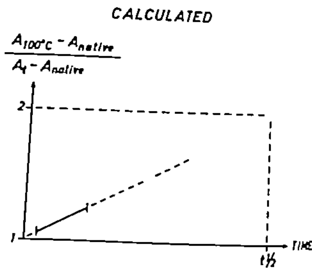


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Since y can be estimated by a_{yt} it applies that

$$a_{yt} \approx y = (A_{100°C} - A_{strom})/(A_o - A_{strom})$$

hich leads to an estimate of A_o given by

$$A_o - A_{strom} = (A_{100°C} - A_{strom}) / a_{yt}$$

$$\text{If } y_t = \frac{A_o - A_{strom}}{A - A_{strom}} = \frac{y}{a_{yt}}$$

is plotted against t, the right-hand side of Fig 1 is obtained The slope of this line becomes

$$\frac{b_{yt}}{a_{yt}}$$

nd the intercept is now 1 The intercept of the line with 1 2 is obtained at time

$$t_{\frac{1}{2}} = 1/\text{slope} = \frac{a_{yt}}{b_{yt}}$$

The concentration of the single-stranded DNA in moles of nucleotides per 1 is calculated from the absorbance at 260 nm at room temperature

$$\frac{A \times 50}{2 \times 331 \times 1000}$$

assuming that 50 µg DNA/ml gives an absorbance of 1 The mono molecular weight for a sodium nucleotide is 331

RESULTS

The ratios 260 nm/280 nm and 260 nm/230 nm of the DNAs isolated before and after CTAB treatment are shown in Tables 2 and 3 The preliminary reassociation and hybridization experiments at varying salt concentrations from 1 to 6 SSC showed that the time for 50 per cent reassociation and consequently $C_ot\frac{1}{2}$ decreased about four-fold by increasing the salt concentration from 1 to 3 SSC (Table 4) The change in the salt concentration from 3 to 6 SSC caused a very slight decrease of $C_ot\frac{1}{2}$ but the homology percentage

TABLE 2 [illegible] nm of the [illegible]

Species	[illegible]
M bovis, BCG	[illegible]
M kansasii	[illegible]
M marinum	[illegible]
M gordonae	1 [illegible]
M avium	1 6[illegible]
M gastri	1 57
M smegmatis	1.88
M phlei	–
M fortuitum	1.88
E coli B	1 81

...action to the latter ... bands remained ... and three h of ... ased density of ... d to be due to ... layers in the ... ses, and is ... the results. ... ple may ... fractions ... *kingae* ... alytical ... lytical ... *ingae* ... was ... nd ... ot

TABLE 3 *Ratio between Absorbance at 260 nm and [illegible] nm of the DNA before and after CTAB Treatment*

Species	260 nm/230 nm Before CTAB	After CTAB
M bovis, BCG	–	2.26
M kansasii	1 28	2 20
M marinum	1 44	2 24
M gordonae	1 20	2 20
M avium	1 27	2 16
M gastri	1 02	2.14
M smegmatis	2 11	2 24
M phlei	–	2.32
M fortuitum	2 17	2.22
E coli B	2 25	2 25

TABLE 4 *Preliminary Results of DNA-DNA Hybridizations with Different Concentrations of DNA SSC and Formamide*

DNA µg/ml	SSC conc	Formamide per cent	$C_ot\frac{1}{2}$ *M phlei*	$C_ot\frac{1}{2}$ *M smegmatis*	$C_ot\frac{1}{2}$ BCG	Homology per cent
40	6	25		0 904	0 447	30 8
40	4	25		1 009	0 612	17 [illegible]
40	3	.5		0 978	0.576	8.9
40		25		1 704	0 966	5 7
40	1	25		4 094	2 151	9 2
40	6	50		1 467	0 907	31.6
40	3	25	0 914			
70	3	25	0 934			
10	3	25	0 955			

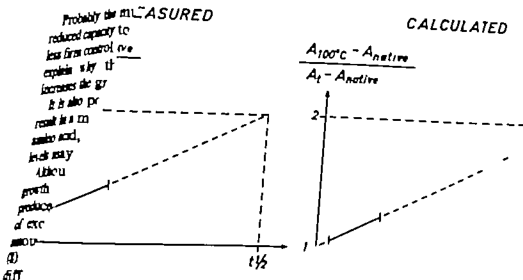


Fig. 1 Kinetics of the DNA DNA hybridization
Absorbance measured at 270 nm of DNA in native condition, at 100°C at time 0 and
—| Part of the experiment used for calculations.
Left side: Measured values.
Right side: Calculated values after extrapolation to A at time 0

Shearing of DNA The DNA was sheared at a concentration of 800 µg/ml in a French pressure cell press at 20 000 p s.i The sedimentation coefficient of the sheared DNA in 1 x SSC (0 15 M sodium chloride, 0 015 M trisodium citrate) was determined by analytical ultracentrifugation. From the extrapolated value

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After some preliminary experiments with salt concentrations from 1 to 6 SSC, DNA concentrations of about 10 20 and 40 µg/ml, and formamide concentrations of 25 and 50 per cent, we decided to perform the hybridizations with 40 µg DNA/ml in 3 SSC and 25 per cent formamide. Formamide is necessary in order to reduce the temperature in the ... tion temperature, 25°C bel... was about 60°C. T_m was ... combination of DNAs was ... experiments, for the var... formamide. The temperat... controlled by thermistor in ... reference and the three DN...

After measurement of ... at 260 nm, the cuvettes w... 100°C and kept there for ... was then changed as quickly ... another oil bath adjusted be... temperature. When the ten... absorbances were measured at ... absorbances in the hybridi... measured at 270 nm because ... formamide is less there than at ...

Calculation of the $C_0t\,½$ s ar... percentages between the DNA of ... on the bases of the absorbances at ... 100°C, and the rate of abs... hybridization The second-order ... experiment is shown in Fig 1 The ... hand part of the figure is

$$y_t = (A_{100^\circ C} - A_{native})/(A \ldots$$

where A_t is the value read at time t ... changeover to the oil bath with t... temperature is chosen as t = 0 The cur... since $A_{100^\circ C}$ is different from A_o, which ... the absorbance of DNA at 25°C below ... This is due to the physical change in the ... temperature decreases. The slope of the l... intercept at the y axis are determined by ... analysis (*Diem & Lentner* 1971) These are de... and a_{yt}, respectively

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$$\text{If } y_1 = \frac{A_0 - A_{native}}{A_t - A_{native}} = \frac{y}{b_{yt}}$$

is plotted against t, the right-hand side of Fig. 1 is obtained. The slope of this line becomes

$$\frac{b_{yt}}{b_{yt}}$$

and the intercept is now 1. The intercept of the line with y_1 2 is obtained at time

$$t_{\frac{1}{2}} = 1/\text{slope} = \frac{b_{yt}}{b_{yt}}$$

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$$\frac{A \times 50}{2 \times 331 \times 1000}$$

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TABLE 2. *Ratio between Absorbance at 260 nm and 280 nm of the DNA before and after CTAB Treatment*

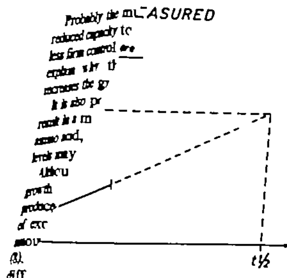
Species	260 nm/280 nm	
	Before CTAB	After CTAB
M. bovis, BCG	1.86	1.88
M. kansasii	1.69	1.88
M. marinum	1.73	1.89
M. gordonae	1.65	1.88
M. avium	1.68	1.87
M. gastri	1.57	1.88
M. smegmatis	1.88	1.92
M. phlei	–	1.89
M. fortuitum	1.83	1.86
E. coli B	1.81	1.81

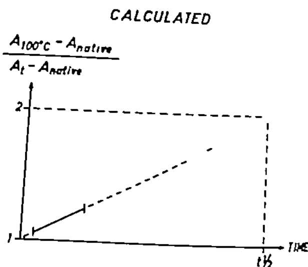
TABLE 3. *Ratio between Absorbance at 260 nm and 230 nm of the DNA before and after CTAB Treatment*

Species	260 nm/230 nm	
	Before CTAB	After CTAB
M. bovis, BCG	–	2.26
M. kansasii	1.28	2.20
M. marinum	1.44	2.24
M. gordonae	1.20	2.20
M. avium	1.27	2.16
M. gastri	1.02	14
M. smegmatis	2.11	4
M. phlei	–	3
M. fortuitum	2.17	2
E. coli B	2.25	2.25

TABLE 4. *Preliminary Results of DNA-DNA Hybridizations with Different Concentrations of DNA, SSC and Formamide*

DNA µg/ml	SSC conc	Formamide per cent	$C_0t_{\frac{1}{2}}$ *M. phlei*	$C_0t_{\frac{1}{2}}$ *M. smegmatis*	$C_0t_{\frac{1}{2}}$ BCG	Homology per cent
40	6	25		0.904	0.447	30.8
40	4	25		1.009	0.612	17.2
40	3	25		0.978	0.576	8.9
40	2	25		1.704	0.966	5.7
40	1	25		4.094	2.151	9.2
40	6	50		1.467	0.907	31.6
40	3	25	0.914			
20	3	25	0.934			
10	3	25	0.955			





1 Kinetics of the DNA DNA hybridization
A Absorbance measured at 270 nm of DNA in native condition, at 100°C, at time 0 and at time t
—| Part of the experiment used for calculations.
Left side: Measured values.
Right side. Calculated values after extrapolation to A at time 0

Shearing of DNA The DNA was sheared at a concentration of 800 μg/ml in a French pressure cell press at 20 000 p.s.i The sedimentation coefficient of the sheared DNA in 1 x SSC (0 15 M sodium chloride, 0 015 M trisodium citrate) was determined by analytical ultracentrifugation. From the extrapolated value

$$(S^{c=0}_{25\,w})$$

a molecular weight of 681 000 daltons (± 5 per cent) was calculated *(Crothers & Zimm* 1965)

Hybridization The reassociation of two bacterial DNAs as compared with the hybridization of the mixture of these, was measured optically in a spectrophotometer The $C_0t\,½$ of a DNA is defined by *Britten & Kohne* (1966) as the concentration of DNA in moles of nucleotides per l multiplied by the time in seconds required for 50 per cent reassociation If two bacterial strains have identical DNAs, the $C_0t\,½$ of the mixture of their DNAs is the same as the $C_0t\,½$ of each single DNA If two bacterial strains have no mutual DNA sequences, the $C_0t\,½$ of the mixture will be the sum of their $C_0t\,½$ s This is described by *Seidler & Mandel* (1971) and expressed in their equation which is transformed by *Bradley* (1973) to homology percentage = 100 + 100 ($C_0t\,½$ A + $C_0t\,½$ B − 2 $C_0t\,½$ M)/ ($C_0t\,½$ A + $C_0t\,½$ B). A and B are the two bacteria to be compared and M the mixture.

After some preliminary experiments with salt concentrations from 1 to 6 SSC, DNA concentrations of about 10 20 and 40 μg/ml, and formamide concentrations of 25 and 50 per cent, we decided to perform the hybridizations with 40 μg DNA/ml in 3 SSC and 25 per cent formamide. Formamide is necessary in order to reduce the temperature in the experiment The hybridization temperature, 25°C below T_m, in the mixture used was about 60°C T_m was determined each time a new combination of DNAs was used and in the preliminary experiments, for the various concentrations of SSC and formamide The temperature in each experiment was controlled by thermistor in a cuvette placed between the reference and the three DNAs, A, B and M

After measurement of the concentration of the DNAs at 260 nm, the cuvettes were heated to between 99°C and 100°C and kept there for 15 minutes The temperature was then changed as quickly as possible by changing to another oil bath adjusted beforehand to the hybridization temperature. When the temperature was reached, the absorbances were measured at 2 minute intervals The absorbances in the hybridization experiments were measured at 270 nm because the influence of the formamide is less there than at 260 nm

Calculation of the $C_0t\,½$ s and of the homology percentages between the DNA of two bacteria was made on the basis of the absorbances at room temperature and 100°C and the rate of absorbance decrease during hybridization The second-order reaction plot of the experiment is shown in Fig 1 The ordinate in the left hand part of the figure is

$$y_t = (A_{100°C} - A_{native})/(A_t - A_{native})$$

where A_t is the value read at time t The time for the changeover to the oil bath with the hybridization temperature is chosen as t = 0 The curve is not correct. since $A_{100°C}$ is different from A_0, which corresponds to the absorbance of DNA at 25°C below T_m at time 0 This is due to the physical change in the DNAs as the temperature decreases The slope of the line and the intercept at the y axis are determined by regression analysis *(Diem & Lentner* 1971) These are designated b_{yt} and a_{yt}, respectively

The average $\bar{y}$ generally lies near 1.3 (start near 1.2, end y near 1.2 + 0.2 = 1.4)

Since

$$\frac{\bar{y}}{b} = t_X + \bar{t},$$

the relative variance of t_X, $V\{t_X\} / t_X^2$ becomes

$$(6) = \frac{\sigma^2}{n} \quad (0.6 + 12 \quad \frac{n-1}{n+1} \quad 25) \quad (1 + \frac{\bar{t}}{t_X})^2$$

The ratio $\bar{t}/t_Y$ lies near 0.3 in the majority of the experiments. With n = 8 the right-hand side then becomes

$$(7) \frac{\sigma^2}{n} \quad (0.6 + 233) \quad 1.3^2$$

The relative variance of t_Y is clearly dominated by the contribution originating from the uncertainty of the slope (compare 233 against 0.6 in (7)). This agrees with the fact that extrapolation for t_X is made on the basis of part of the line.

The formulas for the variance are derived on the assumption that the values observed for y (i.e. A_i) are statistically independent. This can hardly be correct for observations taken at such short time intervals. With intervals of 2 minutes there is no immediate sign of dependence on the plots for the individual experiments, but a numerical control can be made by taking the means of three y values at a time (i.e. ⅓ (y + y₂ + y₃), ⅓ (y₄ + y₅ + y₆), etc.) and using these values in the regression calculation. This has been done for 19 of the experiments. In many of the experiments there are 24 y values for A and B and 36 for M, i.e. 8 averages for A and B and 12 for M. Calculations with the individual values give an estimate of σ^2 at about 25×10^{-4} for A, B and M.

For the means $\sigma^2 \approx 11 \times 10^{-4}$. This is greater than the ⅓ × 25 × 10^{-4} which could be expected on the assumption of independence, thus indicating a weakly positive correlation. If the means are used instead of individual values, there is no need for further correction for correlation. The variance corresponding to $\sigma^2 \approx 11 \times 10^{-4}$ and n = 8 for A and B is then

$$\frac{V\{t\}}{(t)^2} = 5.43 \quad 10^{-4}$$

or a *relative standard deviation of 0.023 (2.3 per cent)*.

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$$V\{H\} = (200?) \times (\frac{M}{A+B})^2 (\frac{V\{M\}}{M^2} + \frac{V}{(A+...}$$

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DISCUSSION

The advantage of optical measurement of DNA-DNA reassociation is that one can avoid radioactive labelling of the DNA and problems with the leaching of DNA from the filters in reassociation on membrane filters. The disadvantage with optical measurement is that only one homology percentage can be determined at a time.

The method used here has proved to be satisfactory in practice. The mean standard deviation for determination of the homology percentages is 5.5 per cent. The calculations have shown that the variance of t_Y is only about one-fifth of the total variance. Presumably the remainder lies primarily in the concentration determinations. These are difficult because several small volumes have to be mixed. Particularly the addition of formamide is a problem since formamide affects the measurement at 260 nm. Furthermore, transfer from one cuvette to another can involve a change in the absorbance for no apparent reason. We were not able to perform the diphenylamine reaction for determination of the DNA concentration of the final mixture of DNA, presumably because of the presence of formamide.

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The addition of 50 per cent formamide instead of 25 per cent increased the reassociation time considerably but had no effect on the homology percentage.

In order to achieve a reasonable rate of reassociation and at the same time avoid a presumably too high homology percentage, 3 SSC and 25 per cent formamide were chosen for subsequent experiments.

Experiments with the DNA of the same bacteria in concentrations of approximately 10 20 and 40 μg/ml in 3 SSC and 25 per cent formamide resulted in unchanged $C_0t\frac{1}{2}$. In order to obtain the largest possible distance between A_0 and A_{term} 40 μg/ml was chosen, since the largest possible absorbance decrease per time during the course of the experiment must give the greatest accuracy in the determination of $t\frac{1}{2}$

The homology percentages of *M smegmatis* ATCC 607 as compared with nine different species of mycobacteria are shown in Table 5. Each value represents the mean of four experiments. The homology percentage between the two *M smegmatis* strains is high. There is a low degree of homology between *M smegmatis* and the other two rapidly growing mycobacteria, and almost no homology between *M smegmatis* and the slowly growing mycobacteria.

TABLE 5 *Homology Percentages of the DNA of M smegmatis ATCC 607 as Compared with the DNA of Nine Different Species of Mycobacteria*

Species	Per cent homology with *M smegmatis ATCC 607*	Standard deviation
M bovis, BCG	11 3	6 1
M kansasii	13 9	4 8
M marinum	11 7	5 2
M gordonae	3 4	7 7
M avium	13 2	5 2
M gastri	8 8	6 8
M smegmatis	96.5	4 2
M phlei	29.9	4 4
M fortuitum	23.3	4 2

Examination of the Statistical Uncertainty in Determination of Homology Percentages

The homology percentage (H) is determined as stated under MATERIAL AND METHODS. The formula given there can be written

$$H = 200 \quad (1 - \frac{M}{A + B}) \text{ where}$$

$$A = (t\tfrac{1}{2} \text{ for A}) \quad C_0 \text{ for A}$$
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The first problem is to find the variance of the $t\frac{1}{2}$ determinations.

As mentioned previously

$$(1)\ t\tfrac{1}{2} = \frac{a_{yt}}{b_{yt}} = \frac{\bar{y} - b_{yt}\ \bar{t}}{b_{yt}} = \frac{\bar{y}}{b_{yt}} - \bar{t} = \frac{\bar{y}}{b} - \bar{t}$$

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$$(3)\ S_t = n \quad \frac{n^2-1}{12} \quad \omega^2$$

Since the slope must lie near $\Delta y/\Delta t$

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$$(5)\ V\{t\tfrac{1}{2}\} = (\frac{\bar{y}}{b})^2 \quad \frac{\sigma^2}{b^2} \quad (\frac{b^2}{n\bar{y}^2} + \frac{12(n-1)^2}{n(n^2-1)} \quad \frac{b^2}{(\Delta y)^2})$$

$$= (\frac{\bar{y}}{b})^2 \frac{\sigma^2}{n} \quad (\frac{1}{\bar{y}^2} + 12 \ \frac{n-1}{n+1} \ \frac{1}{(\Delta y)^2})$$

It has been shown that the rectilinear part of the curve in Fig 1 corresponded to a total change in y of 0 20 for both A B and M (with homology percentages near 0 twice as long an observation time for M is required).

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It has been shown that the rectilinear part of the curve in Fig 1 corresponded to a total change in y of 0 20 for both A B and M (with homology percentages near 0 twice as long an observation time for M is required).

Acta path. microbiol. scand. Sect. B, 86: 77–86, 1978.

DENSITY GRADIENT CENTRIFUG[A]… IN UROGRAFIN OF *MORAXELLA* AND *K*… CELLS AND APPENDAGES

LEIF ODDVAR FRØHOLM and KJELL BØVRE

Methodology Department, National Institute of Public Health, Oslo, Kaptein W. Wilhelmsen… Bakteriologiske Institutt, University of Oslo, Rikshospitalet, Oslo, and Institute of Medical… University of Tromsø, Tromsø, Norway

Frøholm, L. O. & Bøvre, K. Density gradient centrifugation in Urografin of *Moraxella* and *Kingella* cells and appendages. Acta path. microbiol. scand. Sect. B, 86: 77–86, 1978.

Purification of fimbriae (pili) by density gradient banding in Urografin medium was attempted. *Moraxella nonliquefaciens* and *Kingella kingae* fimbriae were of higher density than their cells of origin, but fimbrial fractions obtained by homogenization and differential centrifugation still banded together with presumed outer membrane fragments and some whole cells in Urografin gradients. The cellular density of genetic variants with different fimbriation/competence levels was also studied. For one strain of *M. nonliquefaciens* and two strains of *K. kingae*, cells harvested from agar plates tended to show several bands on isopycnic density gradient centrifugation, with slightly higher general density of fimbriated variants than non-fimbriated. A single density band could be observed with cells from log phase broth cultures of selected strains which showed no distinct difference between fimbriation or competence variants of each strain. Cells of *M. nonliquefaciens* and *M. bovis* showed comparable buoyant densities, whereas those of *K. kingae* had a higher density.

Key words: Buoyant density of bacteria; isopycnic gradient centrifugation; cellular fractionation; fimbriae; pili; *Moraxella*; *Kingella*; Urografin.

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Previous studies in our laboratories have demonstrated several differences between fimbriated and non-fimbriated or sparsely fimbriated variants of *Moraxella*, *Kingella* and *Neisseria* (1, 2, 8, 9, 10, 12, 19). (See also ref. 18.) One important finding was a correlation between fimbriation and competence in genetic transformation (2).

The present paper describes the physical properties of fimbriae and cells of fimbriated and non-fimbriated variants of *Moraxella* and *Kingella* in Urografin gradients, and gives a report of attempts to purify fimbriae by use of such gradients. Urografin was chosen for density gradient experiments because it had been used previously for the separation of pure flagella (6). It would also permit studies with living cells (17, 23, 25) and comparison with studies in *Bacillus subtilis*. In this species, physiologically competent variants were of lower density than the culture as a whole (4, 11, 21).

MATERIALS AND METHODS

Bacterial strains. Moraxella nonliquefaciens 4663/62 N-b and SC-a, and NCTC 7784 N-b and SC-a, *M. bovis* 4 N-b and SC-a, and 5 N-a and SC-b, and *Kingella kingae* (13) 4177/66 N-b and SC-a, and A1702 N-a and SC-a, have all been included in previous investigations on fimbriation and associated characters (1, 2, 8, 9, 12, 19). *M. nonliquefaciens* 7784 SC-c originated as a highly

Six of the measured homology percentages can be compared with corresponding values determined by Bradley (1972 1973) and Gross & Wayne (1970) The results are similar but four of Bradley's homology percentages are somewhat higher than ours These are the homology percentages between M smegmatis and BCG- 11 3 and from 12–24 M marinum 11 7 and 19 M phlei 29 9 and 45 and M fortuitum 23 3 and 37 the highest values being Bradley's results The difference between the methods used is that Bradley uses a higher salt concentration, and he does not mention whether he has purified his DNA for polysaccharides Polysaccharides are apparently a common problem in the production of DNA from mycobacteria, since they were also found by *Hill et al* (1972), even though they used a completely different method.

The preliminary experiments in the present work showed also an increasing homology percentage when the salt concentration became higher than 3 SSC. Thus the difference between *Bradley's* and the present results may be explained by the difference in the salt concentrations *viz.* 6 SSC and 3 SSC It might be that non-specific base-pairing is obtained by using 6 SSC which is avoided at 3 SSC. This aspect has not been examined.

After development of the method, it is intended to use it in solving special problems within the field of mycobacterial taxonomy

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Grateful thanks are due to *Bendt Mansa* Statens Seruminstitut for performing the molecular weight determinations.

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Seidler R J & Mandel, M Quantitative aspects of deoxyribonucleic acid renaturation Base composition, state of chromosome replication, and polynucleotide homologies. J Bact *106* 608–614 1971

Electron microscopy Cells and fimbriae in TS or PBS suspensions were sedimented by centrifugation of 0 2 ml volumes in a Micro centrifuge (Ole Dich, Hvidovre) at 4,000 × *g* for one and ten min, respectively The pellets were suspended in 0 02 ml 0 8 per cent SST and transferred to *grids for electron microscopy* (1) *Samples* from Urografin gradients were usually diluted two to four-fold in destilled water and either centrifuged in the Micro centrifuge, washed once in negative stain solution, and pellets suspended in negative stain solution as above, or centrifuged for 15 min at 1 750 × *g* onto grids in a special holder (7) The holder was disassembled and the grids treated for 15 min with stain solution from beneath, flowing through a filter paper supporting the grid (24)

RESULTS

Characteristics of Urografin gradients The shape of the analytical gradient 1 immediately after preparation can be seen in Fig. 1 A 0.5 ml aliquot of 1 100 g/cm³ density Urografin solution was layered on top of the gradient before drop collection to mimic the actual analysis The effect of 30 and 120 *min* centrifugation after 20 and 18 h diffusion is also illustrated Fractionation by drop collection shows that the gradient after 120 min centrifugation is almost linear between n_D^{20} 1 3680 and n_D^{20} 1 4015 corresponding to the densities of 1 121 g/cm³ and 1 237 g/cm³ respectively As expected, immediate collection of fractions shows that the gradient is stepwise The apparent difference in slope between 30 and 120 min centrifugation may be due to slight differences in total volume or drop size as indicated by a limited volume of the last fraction in the latter experiment. The pattern of cell bands remained indistinguishable between one and three h of centrifugation (not shown). The increased density of top fractions (to the right) is believed to be due to evaporation of water from the surface layers in the tube during the collection of fractions, and is considered to be of no consequence for the results. Residual moisture in the collection needle may explain the lower density of some bottom fractions.

Because of the relatively high density *K kingae* cells banded near the bottom of the analytical gradient 1 (*cf* Fig. 3). For this reason the analytical gradient 2 which gave a band position of *K kingae* near the middle was developed. This gradient was almost linear between 1 165 and 1 265 g/cm³ and was as stable as the analytical gradient 1 (not shown, *cf* Fig. 5).

When post-logarithmic *M bovis* 9 SC cells were suspended in Urografin solution of density 1.244 g/cm³ after the usual washing in 1 100 g/cm³ solution and exchanged for 0.5 ml from the bottom *of the tube, they reached the same position in the* analytical gradient 1 as cells treated in the regular way However some aggregation occurred in the samples started from the bottom of the tubes. Rebanding experiments were also performed and these ensured that an almost identical position was reached in the second gradient.

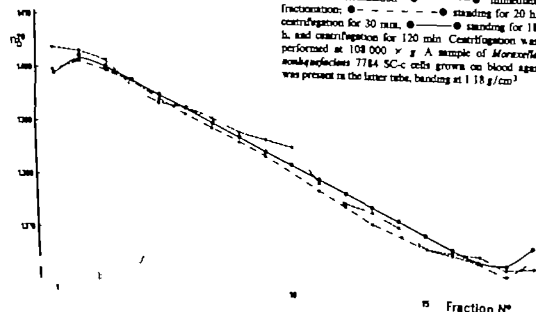


Fig 1 Refractive index changes in the Urografin gradient with time and centrifugation See text for preparation of gradients, and for refractive index values and their determination ●- - - - - -● immediate fractionation; ●— — — — —● standing for 20 h, centrifugation for 30 min, ●———● standing for 18 h, and centrifugation for 120 min Centrifugation was performed at 108 000 × *g* A sample of *Moraxella nonliquefaciens* 7784 SC-c cells grown on blood agar was present in the latter tube, banding at 1 18 g/cm³

Electron microscopy. Cells and fimbriae in TS or PBS suspensions were sedimented by centrifugation of 0.2 ml volumes in a Micro centrifuge (Ole Dich, Hvidovre) at 18,000 × *g* for one and ten min, respectively. The pellets were suspended in 0.02 ml 0.8 per cent SST and transferred to grids for electron microscopy (1). Samples from Urografin gradients were usually diluted two to four-fold in distilled water and either centrifuged in the Micro centrifuge, washed once in negative stain solution, and pellets suspended in negative stain solution as above, or centrifuged for 15 min at 1 750 × *g* onto grids in a special holder (7). The holder was disassembled and the grids treated for 15 min with stain solution from beneath, flowing through a filter paper supporting the grid (24).

RESULTS

Characteristics of Urografin gradients. The shape of the analytical gradient 1 immediately after preparation can be seen in Fig. 1. A 0.5 ml aliquot of 1.100 g/cm³ density Urografin solution was layered on top of the gradient before drop collection to mimic the actual analysis. The effect of 30 and 120 min centrifugation after 20 and 18 h diffusion is also illustrated. Fractionation by drop collection shows that the gradient after 120 min centrifugation is almost linear between n_D^{20} 1.3680 and n_D^{20} 1.4015 corresponding to the densities of 1.121 g/cm³ and 1.237 g/cm³ respectively. As expected, immediate collection of fractions shows that the gradient is stepwise. The apparent difference in slope between 30 and 120 min centrifugation may be due to slight differences in total volume or drop size as indicated by a limited volume of the last fraction in the latter experiment. The pattern of cell bands remained indistinguishable between one and three h of centrifugation (not shown). The increased density of top fractions (to the right) is believed to be due to evaporation of water from the surface layers in the tube during the collection of fractions, and is considered to be of no consequence for the results. Residual moisture in the collection needle may explain the lower density of some bottom fractions.

Because of the relatively high density *K. kingae* cells banded near the bottom of the analytical gradient 1 (*cf.* Fig. 3). For this reason the analytical gradient 2 which gave a band position of *K. kingae* near the middle was developed. This gradient was almost linear between 1.165 and 1.265 g/cm³ and was as stable as the analytical gradient 1 (not shown, *cf.* Fig. 5).

When post-logarithmic *M. bovis* 9 SC cells were suspended in Urografin solution of density 1.244 g/cm³ after the usual washing in 1.100 g/cm³ solution and exchanged for 0.5 ml from the bottom of the tube, they reached the same position in the analytical gradient 1 as cells treated in the regular way. However some aggregation occurred in the samples started from the bottom of the tubes. Rebanding experiments were also performed and these ensured that an almost identical position was reached in the second gradient.

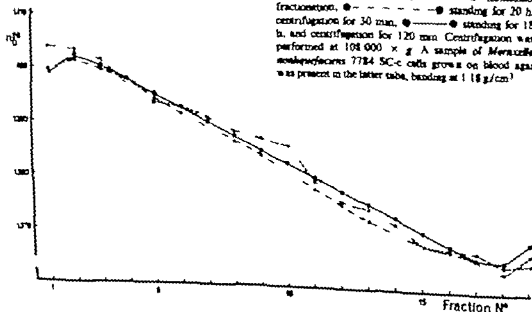


Fig. 1. Refractive index changes in the Urografin gradient with time and centrifugation. See text for preparation of gradients, and for refractive index values and their determination. ●-------● immediate fractionation, ●– – – – –● standing for 20 h, centrifugation for 30 min, ●———● standing for 18 h, and centrifugation for 120 min. Centrifugation was performed at 108 000 × *g*. A sample of *Moraxella nonliquefaciens* 7784 SC-c cells grown on blood agar was present in the latter tube, banding at 1.18 g/cm³.

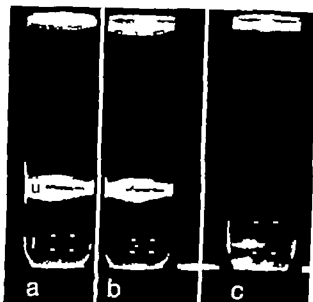


Fig 2 Separation of *Kingella kingae* fimbriae from whole cells. A combined supernatant fraction (See Materials and Methods) of blood agar grown *K. kingae* 4177/66 SC-a (3 5 ml) was layered on top of a preparative Urografin gradient (see text) Boundaries between gradient layers as prepared are marked on tubes a and b which are parallels. The upper band (u) contains mostly cells, while the lower band (l density 1 24 g/cm^3) is a fimbrial band with many cells. In Fig. 2 c the 1 24 g/cm^3 zone of the previous gradients (pooled material) was diluted with distilled water to a density of 1 200 g/cm^3 and layered over the following gradient. 1 217 g/cm^3 Urografin, 0 3 ml 1 240 g/cm^3 0 6 ml and 1 262 g/cm^3 0 3 ml Centrifugation was performed at 192,000 × g The upper and lower limits of the initial 1 240 g/cm^3 layer are indicated.

Densities concentration and purification of fimbriae from M nonliquefaciens *and* K. kingae. *K kingae* fimbrial fractions were found to have an apparent equilibrium density in Urografin of about 1 24 g/cm^3 This is only slightly higher than that of the cells (see below) Fig. 2 a and b show the separation of a combined supernatant fraction of *K kingae* 4177/66 SC-a into an upper layer consisting mainly of cells (u) and a concentrated fimbrial suspension in the lower part of the tube (l) Electron microscopy of the fimbrial band showed that this contained some whole cells and presumably outer membrane fragments in addition to fimbriae (see below) Although the enrichment of fimbriae in this fraction was considerable and the fraction useful as a concentrate it proved impossible to get rid of the contaminants by rebanding in Urografin (Fig. 2 c), in CsCl gradients or by isoelectric focusing (7) The subsequent purification attempts tended to give two or three bands, each of which were shown by electron microscopy to

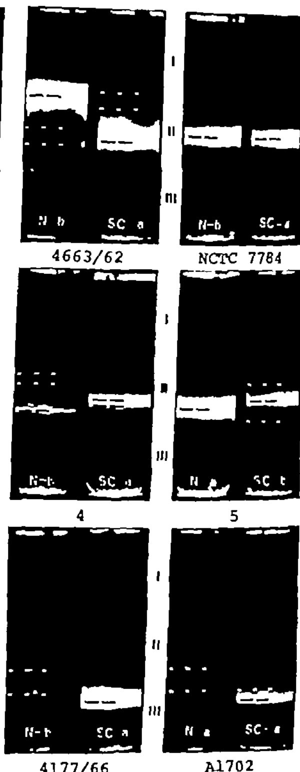


Fig 3 Density distribution in Urografin gradients of agar grown fimbriation variants of *Moraxella* and *Kingella* The «four component analytical gradient *I* was used Cells were grown on blood agar plates (1) for two days See text for details and further references Roman numerals I II, and III indicate the regions of densities 1 125 1 175 and 1 225 g/cm^3 respectively In this range the gradient is almost linearly increasing in density (Fig 1) One N and one SC variant of each strain as indicated on the lower part of the tubes (grown strictly in parallel) were used Each distinctly visible band is indicated with a dotted tracing on the tube

contain a mixture of cells, cell fragments and fimbriae differing little in appearance. *M. nonliquefaciens* 7784 SC-c gave similar results. Although the fimbriae of this strain usually had an equilibrium density of 1.22 g/cm^3 which is distinctly higher than that of the cells of this species (see below), cellular fragments and some whole cells always contaminated fimbrial fractions. In addition, the fimbrial density showed some variation from experiment to experiment (*cf.* Fig. 2 c), presumably because of the presence of varying amounts of contaminants.

Densities of bacterial cells from different media and stages of growth. Fig. 3 shows the distribution of whole cells in the analytical gradient 1 of two-day old blood agar cultures of N and SC variants of *M. nonliquefaciens*, *M. bovis* and *K. kingae*, two strains of each. Only *M. nonliquefaciens* 7784 gives rise to one band, which is of similar density for both variants. The other strains show up to three bands, and some variants show broad bands indicating a wide density distribution of cells in a single band. The *K. kingae* cells occupy positions close to the bottom of these gradients, whereas the *M. nonliquefaciens* and *M. bovis* cells are located in the middle or higher. *M. bovis* is possibly a little more dense than *M. nonliquefaciens*. In both of the *K. kingae* strains and in *M. nonliquefaciens* 4663/62, the bulk of the SC cells are more dense than the N type of cells. In *M. bovis*, almost the same density is observed for the two variants, perhaps slightly less for the SC type. These differences may be due to the fimbriae having density other than that of the cells (*i.e.* higher in *M. nonliquefaciens* and *K. kingae*). However, further studies disclosed a considerable variation between parallel experiments and also some variation related to the type of medium and to the age of the culture. Consequently, some additional experiments were performed (Figs. 4 and 5).

Fig. 4 shows that *M. nonliquefaciens* 7784 SC-c cells form one band under several different growth conditions, but with somewhat variable density. Tubes d and f illustrate a relatively broad distribution of cells in old cultures and tube e the narrow single band of density 1.195 g/cm^3 generally found in early log phase cultures of this strain. Tube g shows a post-logarithmic culture similar to f but with two bands. The lower band (marked h) was studied by electron microscopy as were the bands u and l of tube d (see below). In additional experiments (not shown) logarithmically growing *M. nonliquefaciens* 4663/62 and 7784 gave a single band of density about 1.19 g/cm^3 regardless of whether fimbriated or non-fimbriated variants were studied. However, both types of strain 4663/62 variants revealed a rather broad band also under these conditions. *M. bovis* was not studied extensively in this way, but logarithmically growing cells

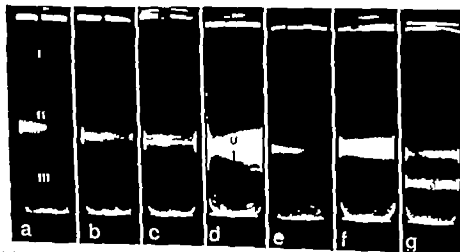


Fig. 4. Density gradient variation of *Moraxella nonliquefaciens* with different growth conditions. The analytical Urografin gradient 1 was used. In a, b and c *M. nonliquefaciens* 7784 SC-c was grown on solid media for 18 h before harvesting: a growth on blood agar medium, b on fresh Mueller Hinton + Yeast Extract agar, c on a three week old batch of the latter medium, d blood agar growth for 44 h (cells from the zones u and l were studied by electron microscopy, see Fig. 6 b and c), e early logarithmic and f early post-logarithmic growth in broth (same culture) and g separate broth culture, early post-logarithmic growth. Cells from the lower granular layer (h) seen in tube g were studied by electron microscopy (Fig. 6 d). See Materials and Methods for details. Density regions corresponding to 1.125, 1.175 and 1.225 g/cm^3 are indicated on tube a with Roman numerals I, II, and III, respectively.

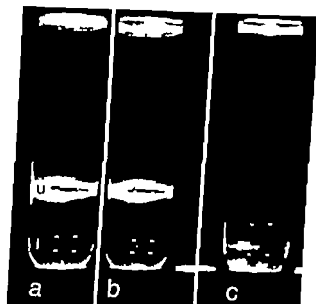


Fig 2 Separation of *Kingella kingae* fimbriae from whole cells. A combined supernatant fraction (See Materials and Methods) of blood agar grown *K kingae* 4177/66 SC-a (3 5 ml) was layered on top of a preparative Urografin gradient (see text). Boundaries between gradient layers as prepared are marked on tubes a and b which are parallels. The upper band (u) contains mostly cells, while the lower band (l, density 1 24 g/cm^3) is a fimbrial band with many cells. In Fig. 2 c the 1 24 g/cm^3 zone of the previous gradients (pooled material) was diluted with distilled water to a density of 1 200 g/cm^3 and layered over the following gradient. 1 217 g/cm^3 Urografin, 0 3 ml 1 240 g/cm^3 0 6 ml and 1 262 g/cm^3 0 3 ml. Centrifugation was performed at 192,000 × g The upper and lower limits of the initial 1 240 g/cm^3 layer are indicated

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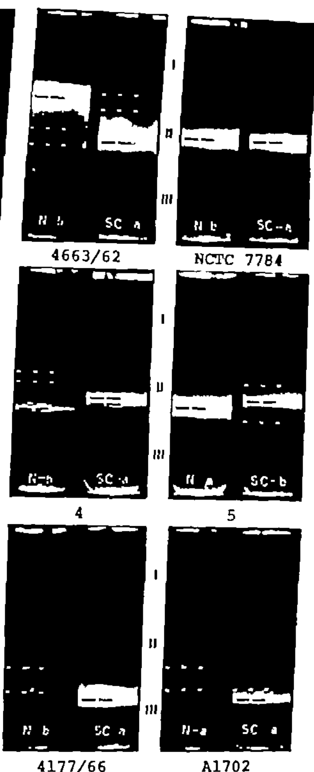
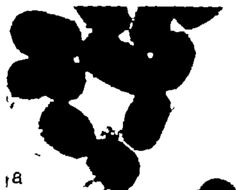
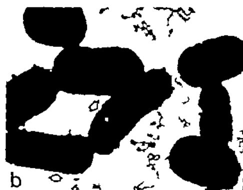


Fig 3 Density distribution in Urografin gradients of agar grown fimbriation variants of *Moraxella* and *Kingella* The *four component analytical gradient* 1 was used Cells were grown on blood agar plates (1) for two days See text for details and further references Roman numerals *I II* and *III* indicate the regions of densities 1 125 1 175 and 1 225 g/cm^3 respectively In this range the gradient is almost linearly increasing in density (Fig 1). One N and one SC variant of each strain as *indicated on the lower part of the tubes* (grown strictly in parallel) were used. Each distinctly visible band is indicated with a dotted tracing on the tube


a


b


c


d


e


f

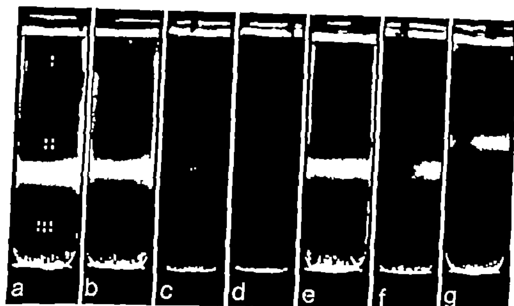


Fig 5 Density gradient centrifugation of *Kingella kingae* 9076/70 N-b and SC-b variants. The analytical Urografin gradient 2 was used. Tubes a to e show experiments with cells grown in broth: a early logarithmic phase, and b middle logarithmic phase (one experiment), c early logarithmic phase and d middle logarithmic phase (another experiment), all with the N-b variant, and e an experiment with the SC b variant (early logarithmic phase). For comparison tubes f and g show the SC b variant harvested from blood agar after incubation for 20 h and 70 h, respectively. See Materials and Methods for composition of media and other details. Tube f contains a glass bead of density 1 23 g/cm³. Density regions corresponding to 1 165, 1 215 and 1 265 g/cm³ are indicated on tube a with Roman numerals I, II and III (dotted), respectively

of strain 9 SC gave a single, broad band of density *1 20 g/cm³ which is only a little higher than the* density of the *M nonliquefaciens* cells studied.

Fig 5 shows the apparently fimbriation independent density of approximately *1 23 g/cm³ of K kingae* 9076/70 cells, similar to the density of strain 4177/66 SC a shown in Fig 2. A single band of the same density was found for strain A1702 N-a and SC a when grown logarithmically in broth (not shown). Fig 5 a and b show the occasional occurrence of several bands in *K kingae* also when growing logarithmically and Fig. 5 g shows a displacement towards lower cell density with age of blood agar cultures.

Electron microscopy of material collected from gradients In general it was difficult to study gradient material by electron microscopy. The material had to be washed. The single band of logarithmically growing *M nonliquefaciens* 7784 SC-c broth cultures contained fimbriated, single, rod-shaped cells and *diplobacilli* in a well-preserved form by either method of preparation (Fig 6 a). Two-day old agar plate cultures of this clone *showed cells that were apparently more prone to* plasmolysis, with no difference in this respect or as regards fimbriation from cells of slightly different density (Fig 4 d, bands u and l Fig 6 b and c). The light areas indicated by open arrows in Fig 6 b and c are thought to represent spaces of plasmolysis in the cells. The granular high density band (Fig. 4 g, band h) sometimes observed in older cultures, was found to contain aggregated and lysed cells (Fig. 6 d).

Fig 6 e and f show the network of fimbriae in the fimbrial band of *K kingae* 4177/66 SC-a (Fig 2) containing also some cells and cell fragments

Fig 6 Electron micrographs of samples from Urografin gradients. a Logarithmically growing cells of *Moraxella nonliquefaciens* 7784 SC-c sampled from a single band of density 1 195 g/cm³. Preparation by centrifugation onto the grid (7) washed and stained with 0 8 per cent SST (see *Materials and Methods*) through a filter paper supporting the grid 10,000 ×. b The upper part of a broad band of *M nonliquefaciens* 7784 SC-c (Fig. 4 d, band u). Preparation as a 10 000 ×. c The lower part of the broad band studied in b, treated in parallel (Fig 4 d band l) 10 000 ×. The open arrows in b and c point to areas of the cells with reduced staining which may represent plasmolysis. d The lower band (h) of Fig 4 g. A drop of the sample was placed on the grid for two min and the surplus liquid removed by touching the edge with filter paper. Washing and staining with 0 8 per cent SST 10,000 ×. e. The fimbrial band of *Kingella kingae* 4177/66 SC-a from a preparative Urografin gradient (similar to Fig 2 a and b lower band) washed once in 0 8 per cent SST by centrifugation and subsequently stained with 0 8 per cent SST 10 000 ×. f Same preparation as Fig 6 d. 50 000 ×


a
b
c
d
e
f

The electron microscopy data in future experiments should be supplemented by studies of sections of fixed and embedded material which might permit better evaluation of the cell content in such fractions and of the cellular morphology

DISCUSSION

Centrifugation procedure Renografin and Urografin have been used previously to separate different fractions of *e g B subtilis* spores (23), *Mycoplasma laidlawii* (13) and *Coxiella burneti* (25) cells. Iodinated density gradient media have a wide application, as discussed recently (14). The main advantage of using Urografin and similar iodinated media is probably the ease with which different density classes of cells may be obtained in a viable state.

The type of density gradient used does not give a stable slope like a CsCl gradient which is self established under the influence of high centrifugal forces for about 70 h (*cf* ref 5) The shape of the preformed linear Urografin gradient used changes continuously but slowly whether it is centrifuged or not. The present method is only useful when a standardized procedure is followed. The presence of washed bacteria does not seem to influence seriously the refractive index of the gradients (*cf* Fig 1) Hence, collection of material by puncturing the tube from the side may allow rapid establishment of banding density

Separation of fimbriae from other cell material Fimbriae strongly tend to form net like aggregates, which may capture whole cells as well as smaller fragments (Fig. 6 e, f) When, as in *K kingae* the difference between the banding density of the cells (1 23 g/cm^3) and that of the fimbriae (1 24 g/cm^3) is small separation is complicated (only partial). The difficulty of removing *K kingae* cells from their own fimbriae might possibly be overcome by banding in a medium of lower osmotic activity (see below) In *M nonliquefaciens* one would expect the cells (density 1 19 g/cm^3) to band away from their fimbriae (density 1 22 g/cm^3) if the aggregates of lysed cells of about the latter density (Fig 4 g) can be avoided However cells with fimbriae remaining may still contaminate fimbrial fractions

Parallelism has been found between the density of cells and fimbriae so that both are higher in *K kingae* than in *M nonliquefaciens* This may reflect a different fimbrial structure, but may also be explained by complexes with subcellular material which might be of lower density when derived from *M nonliquefaciens* Complexes of different composition might give fimbrial bands of varying density

Flagella were found to give several additional minor bands in both Renografin and CsCl, and this was explained as complex formation (6).

In addition to Urografin density gradient centrifugation, further means are evidently required to ensure complete separation of fimbriae from other cell material Also the true isopycnic density of fimbriae in Urografin is not yet established with certainty due to the incomplete separation.

Density characteristics of whole bacteria. Based on the complex pattern of bands often found by density gradient centrifugation of whole cells from blood agar culture, it has been thought previously that the density distribution of *Moraxella* and *Kingella* variants is not clearly and consistently related to fimbriation or competence in genetic transformation (8). The present experiments show that the density distribution may be less complex for logarithmically growing cells. Only a few selected strains and variants of two species have been examined in this manner but no significant density difference was found between the competent (SC) and the incompetent or lowcompetent (N) variant of the same strain. The sampling of cells by puncturing the side of a tube has permitted subcultivation without contamination with other organisms. No difference has been observed in colonial morphology of cells subcultivated on blood agar from different bands of the same variant.

Thus, our results appear to differ from those found in *B subtilis* where physiologically competent variants had a lower density than incompetent ones (4 11 21)

In *Neisseria meningitidis*, a partial separation between competent and incompetent cells was found within one band after centrifugation in a 5 to 40 per cent (w/v) sucrose gradient at 4 000 rev/min for 10 min at 4° C. The competent cells were enriched in the light density zone, and it was suggested (16) that the difference in sedimentation is connected with the difference in fimbriation or membrane structure observed in this species (10 15). This separation was based on sedimentation velocity and differs from the present method. It remains to be shown whether *Moraxella* and *Kingella* competence variants can be fractionated in a sucrose gradient

Bacterial cells might change density depending upon the medium and growth rate (20) This may partly explain the changes in density distribution found occasionally (see Fig. 4 a – g and Fig. 5 f and g). The occurrence of more than one density band may thus be interpreted as the result of general growth-physiological variation. Several of these strains have been described as pleomorphic, and such size/form variants might partly explain a tendency towards density variation. The occurrence

of lysed and aggregated cells explained a heavier than usual cellular band in one instance during the present series of experiments (Fig. 4 g).

Although the present observations with log phase cultures show that the occurrence of fimbriae is of minor importance as a determinant of overall cellular density, the experiments with blood agar cultures (Fig. 3) may be taken to indicate that fimbriation slightly increases the cellular density of some *M. nonliquefaciens* and *K. kingae* strains. The difference observed between N and SC type cells from blood agar cultures may also be associated with differential effects of growth-physiological factors of the type mentioned above.

The present study indicates that cellular density may be used to characterize genera and species, and Urografin can be used for this purpose. *Wiebe et al.* (25) have compared the separation of two cell types of *C. burneti* in CsCl, sucrose, Renografin and Ficoll. Separation was obtained in the three first mentioned media at osmotic pressures in the cellular bands of approximately 90, 40 and 20 atmospheres, respectively. They found an increase of banding density with higher osmotic pressure. Thus, the rather low osmotic pressure of Renografin was still associated with an increased banding density of whole cells (25). Hence it is difficult to compare directly the banding densities obtained in the present experiments with the results of cell density determination by refractometric methods or sedimentation in organic solvents (26).

Future experiments may include examination of the viability and competence in genetic transformation of cells from different density bands of agar grown cells.

Technical assistance was given by Miss *Sissel Dr...* Mrs. *Lise Hamaldsen*, Mrs. *Lesleman Fredriksen*, Mrs. *Lydia Haggeth* and Miss *Marit Simme*. The Digital Density Meter was made available by Mr. *Fred Jensen* of Norges Apotekerforening. Mr. *Kenn Hagen Antonsen* developed the grid holder for centrifugation and made the tube-puncturing device. We are much indebted to each of them.

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of lysed and aggregated cells explained a heavier than usual cellular band in one instance during the present series of experiments (Fig. 4 g).

Although the present observations with log phase cultures show that the occurrence of fimbriae is of minor importance as a determinant of overall cellular density the experiments with blood agar cultures (Fig. 3) may be taken to indicate that fimbriation slightly increases the cellular density of some *M. nonliquefaciens* and *K. kingae* strains. The difference observed between N and SC type cells from blood agar cultures may also be associated with differential effects of growth-physiological factors of the type mentioned above.

The present study indicates that cellular density may be used to characterize genera and species, and Urografin can be used for this purpose. *Wiebe et al.* (25) have compared the separation of two cell types of *C. burnetii* in CsCl, sucrose, Renografin and Ficoll. Separation was obtained in the three first mentioned media at osmotic pressures in the cellular bands of approximately 90, 40 and 20 atmospheres, respectively. They found an increase of banding density with higher osmotic pressure. Thus, the rather low osmotic pressure of Renografin was still associated with an increased banding density of whole cells (25). Hence it is difficult to compare directly the banding densities obtained in the present experiments with the results of cell density determination by refractometric methods or sedimentation in organic solvents (26).

Future experiments may include examination of the viability and competence in genetic transformation of cells from different density bands of agar grown cells.

Technical assistance was given by Miss *Sissel Dyrnes*, Mrs *Lise Haavaldsen*, Mrs *Veslemøy Fredriksen*, Mrs *Vigdis Hegerli* and Miss *Marit Steine*. The Digital Density Meter was made available by Mr *Fred Jensen* of Norges Apotekerforening. Mr *Knut Hagen Andersen* developed the grid holder for centrifugation and made the tubepuncturing device. We are much indebted to each of them.

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CROSS-REACTIONS BETWEEN *NEISSERIA MENINGITIDIS* AND TWENTY-SEVEN OTHER BACTERIAL SPECIES

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Cross-reactions between antigens from *N. meningitidis* and antigens from 27 other bacterial species from clinical isolates representing 20 different genera were studied by quantitative immunoelectrophoretic methods. A water-soluble *N. meningitidis* antigen preparation and a corresponding pooled rabbit antiserum regularly presenting 48 immunoprecipitates were used as reference system. Antigens from 16 other bacterial genera cross-reacted with one to four *N. meningitidis* antigens, whereas antigens from six other *Neisseria* species cross-reacted with 37 to 48 *N. meningitidis* antigens. Antigens from only three bacterial genera (*Streptococcus*, *Staphylococcus* and *Bacteroides*) did not show any cross-reactions. The degree of cross-reactivity between the antigens were 25–100% as judged by absorption of antibodies. Three of the cross-reacting antigens reacted with normally occurring precipitating antibodies in serum from humans and rabbits.

Key words: *Neisseria meningitidis*, cross-reactivity, quantitative immunoelectrophoresis.

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Accepted as submitted 29 x 77

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intermediate gel between first and second dimension electrophoresis of Ref-ag against Ref-ab (absorption of antibodies in situ). Each analytical series of immunoelectrophoresis was repeated at least twice using different antigen/antibody ratios to confirm the reactions of identity or partial identity between the antigens (1, 2). The degree of cross-reactivity between antigens was estimated after absorption of antibodies in situ according

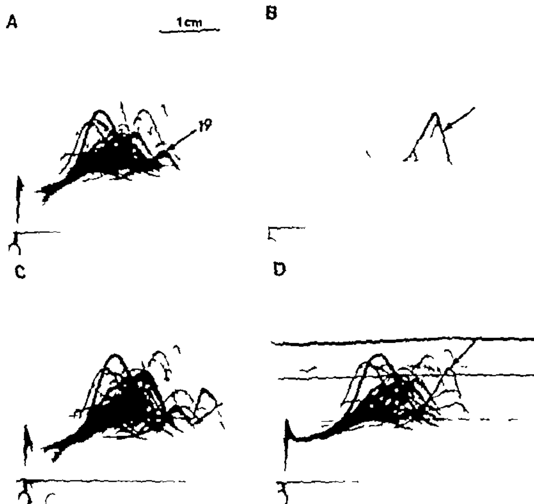


Fig. 1. Comparison of antigens from *Yersinia enterocolitica* and *Neisseria meningitidis* (Ref-ag) using a pooled rabbit antiserum against *N. meningitidis* (Ref-ab).

A. Crossed immunoelectrophoresis of Ref-ag against Ref-ab. Saline in the intermediate gel. Fourty-eight precipitates were seen in this reference pattern. Precipitate no. 19 is indicated by an arrow.

B. Crossed immunoelectrophoresis of *Y. enterocolitica* antigens against Ref-ab. Saline in the intermediate gel. Three distinct precipitates are seen. The precipitate corresponding to no. 19 in the reference pattern is indicated by an arrow.

C. Tandem-crossed immunoelectrophoresis of Ref-ag and *Y. enterocolitica* antigens against Ref-ab. Saline in the intermediate gel. A reaction of partial identity can be seen between one *Y. enterocolitica* antigen (the anodic) and *N. meningitidis* no. 19. The arrow indicates the spur which is partially displaced, indicating a close relationship between the two corresponding antigens (ref. 2). Compare this plate with Fig. 1 A and B.

D. Crossed-line immunoelectrophoresis of Ref-ag against Ref-ab with *Y. enterocolitica* antigens in the intermediate gel (absorption of antibodies *in situ*). The area of precipitate no. 19 has increased considerably (arrow). Compare this plate with Fig. 1 A, B and C.

(Technical: First dimension electrophoresis: anode to the right; second dimension electrophoresis: anode at the top. Staining: Coomassie Brilliant Blue.)

described previously (6 11 13). The remaining *Neisseria* and *Branhamella* species, kindly provided by Dr *Inga Lind* Statens Seruminstitut, were cultured on solid media and suspended in distilled water The antigens were obtained by sonication as described previously (7). The colloid concentration of these antigens were 11 7–59 7 g/l (mean 29 9 g/l) measured by refractometry with human IgG as standard.

Immunoelectrophoretic methods Antigens from each of the 27 bacterial species were compared with the meningococcal Ref-ag by means of a series of quantitative immunoelectrophoreses. Each antigen preparation was run against Ref-ab: 1) in crossed immunoelectrophoreses (23), 2) in tandem-crossed immunoelectrophoresis with Ref-ag (17), and 3) in crossed line immunoelectrophoresis (18) with the antigen in question included in the

TABLE I *Cross reactions between* Neisseria meningitidis *and Other Bacterial Species. The Numbers Signify the Cross reactive Antigens in the Reference System The Number of Strains Tested and the Group Type or Collection Numbers Are Given in Parentheses*

	Cross-reactive antigens and percentage of cross-reactivity			
	100%	100%–≧75%	75%–≧50%	50%–≧25%
Staphylococcus aureus (4 from each of 4 phage groups)				
Streptococcus pyogenes (1 group A)				
Streptococcus faecalis (1)				
Streptococcus pneumoniae (1)				
Bacillus subtilis (1)			19	38
Corynebacterium species (1)				19
Clostridium Welchii (1)				19,38
Haemophilus influenzae (1 type b)		19,38		22
Bordetella pertussis (4 St. 3803–3825–3843–3860)		19		22,38 48
Escherichia coli (2 rough, type O21 H27)			19	22
Salmonella typhi (1)		19		22,38,48
Shigella sonnei (1)			19	22,38
Klebsiella pneumoniae (type 35)			19	38
Serratia marcescens (1)		19		38 48
Proteus mirabilis (1)		19		22,38 48
Yersinia enterocolitica (1 type 3)		19		22,38
Flavobacterium meningosepticum (1)				19
Bacteroides fragilis ss thetaiomicron (1 VP15)				
Vibrio cholerae (1 classica Inaba)		19		38
Pseudomonas aeruginosa (4 O groups 3 5 6 11)		19		22,38 48
Branhamella catarrhalis (2, ATCC 8193 NCTC 4103)				19,22
Neisseria gonorrhoeae (4 ss 19827/40 ss 11413/40 ATCC 11688 ATCC 11689)	42 antigens[a]	55	46	59
Neisseria lactamicus (1 ATCC 23970)	47 antigens[b]		53 55	
Neisseria flavescens (1 ATCC 13120)	36 antigens[c]			54 58 59
Neisseria sicca (1 ATCC 9913)	44 antigens[d]		53	48
Neisseria perflava (1 ATCC 10555)	41 antigens[e]		53	48,55
Neisseria subflava (1)	43 antigens	20,22		

a Three *N meningitidis* antigens (no 21 40 58) were not cross-reactive Antigen no 28 was not cross-reactive with strain ss 19827/40 and no 53 was not cross reactive with strain ATCC 11688

b One antigen (unidentified) was not cross-reactive

c Ten antigens (no 8 8A 4 28 30 41 45 48 54 58) were not cross-reactive

d Antigen no 51 was not cross-reactive.

e Five antigens (no 15 21 25 37 58) were not cross-reactive.

DISCUSSION

Cross-reactions between meningococci and other bacteria have been extensively studied with regard to the capsular polysaccharides (4, 5, 22), but to a much lesser extent as regards other antigens (16, 21). The present study does not pretend to study cross-reactions to meningococcal polysaccharides, since the relative titre in the Ref-ab of antipolysaccharide antibodies was inconveniently low for this purpose. Emphasis has been laid on other antigens, and as much as 48 antigens were found to cross-react with other *Neisseria* species.

Comparisons within the Neisseria Genus

Many taxonomic studies of *Neisseria* have differentiated between »true« and »false« *Neisseria* (10); in our study the »false« *Neisseria* is called *Branhamella* in accordance with Bergey's Manual (20). The present serological study strongly supports the distinctions between *Branhamella* and *Neisseria* on the generic level, since only two of the *Branhamella* antigens cross-reacted with *N. meningitidis*, whereas the other *Neisseria* species cross-reacted very extensively with *N. meningitidis*.

A common biochemical subdivision of *Neisseria* species is into saccharolytic and non-saccharolytic subgroups. The *Neisseria* species examined here all belong to the saccharolytic group, with the exception of *N. flavescens* which is non-saccharolytic, indicating a more remote relationship to the other *Neisseria* species. This was confirmed in the present serological study where *N. flavescens* presented the fewest number of cross-reacting antigens to *N. meningitidis* (Table 1, Fig. 2).

According to our results, none of the 48 antigens in the Ref-ag was found to be specific for *N. meningitidis*, since *N. subflava* showed cross-reactions with all the 48 antigens. However, it should be emphasized that 15 antigens in the Ref-ag preparation have not been examined for cross-reactions to other species, and among these antigens are the group-specific polysaccharides. It is also important to notice that the taxonomic implications of the present results cannot be evaluated until several strains of each species are included in the study, and antisera against these other strains are raised and analysed according to the principles used in this work. Such studies are in progress in our laboratory.

Comparisons with Other Genera

Four *N. meningitidis* antigens (nos. 19, 22, 38 and 48) were found to cross-react with a wide range of other bacterial genera. One of the antigens, no. 19, has been described previously using a *P. aeruginosa* or *B. pertussis* antigen system (11, 12). Some of the other cross-reactive antigens have been identified tentatively with antigens from these two previously described systems. The distribution of these antigens in the various bacteria, as revealed by the present reference system, is practically the same as the distribution found with the two other antigen systems that had been prepared in the same manner as the present study (11, 12).

Antigen no. 19, which is the antigen found in most other bacterial genera, has been shown to correspond to the common antigen or high motility antigen described in *Escherichia coli* by *Kaijser* (15, 16). This antigen is probably different from the common bacterial antigen described by *Kunin* (19), which does not occur in *P. aeruginosa* or *B. pertussis*.

Robbins et al. (21) has also described an antigen found in three *E. coli* strains, which was precipitated by antisera to meningococcal groups A and C. This antigen might correspond to antigen no. 19 or one of the other two antigens found to cross-react with *E. coli*.

Characterization of the Cross-reactive Antigens

Supplementing investigations (*Høiby*, unpublished results) on the cross-reactive antigens have shown that these are heat-labile proteins which are probably not surface antigens. As regards antigen no. 19, the results of *Kayser* (16) showed that the common antigen corresponding to the meningococcal antigen no. 19 is located at the inner part of the bacterial cell wall. None of the four cross-reactive antigens was related to the Forssman antigen or to the endotoxin that has tentatively been identified as antigen no. 55. Nor were they shown to react with the C-reactive protein, in accordance with previous results concerning the *P. aeruginosa* and *B. pertussis* systems (11, 12).

When Ref-ag was assayed in the Limulus test commonly used for detection of endotoxin activity, the test became positive by clotting, and antigens nos. 19 and 22 were found in the clot together with endotoxin (antigen no. 55) (*Høiby & Tvede*, unpublished results). However, besides endotoxin, the *Limulus* lysate has been shown also to react with proteins and polynucleotides (3).

Finally, it has been found that the wide distribution of antigens nos. 19, 22 and 48 is reflected in the occurrence of corresponding precipitating antibodies in sera from nearly all normal human subjects (8), as well as from rabbits (7). *Kayser* (16) also found antibodies against the common antigen (corresponding to our meningococcal antigen no. 19) in rabbits and showed that these antibodies had no protective effect against

to the following principles. The percentage increase of the enclosed area of a *given precipitate after absorption* of antibodies *in situ* is established by comparison with a series of four immunoplates containing 100, 75, 50 and 25% respectively of the original concentration of Ref-ab as described previously (11, 12). In this way the degree of cross reactivity can be expressed as 0, 25–50, 50–75, 75–100 and 100% respectively. Absorption of less than 25% is considered insignificant due to the analytical variation of the present method (14).

The first dimension electrophoresis of the antigens was run with 2 µl, 1 µl, and 1 µl diluted 1:2 or 1:4 if necessary. The intermediate gel of crossed-line immunoelectrophoresis contained the antigen in question undiluted (33.3 µl/cm²) or in two-fold dilutions hereof or 0.9% saline as control. The reference gel contained Ref-ab (5 µl/cm² or 2.5 µl/cm²). Immunoplates which were compared by these methods were always run simultaneously.

RESULTS

The results of the comparison of antigens from 27 different bacterial species representing 20 different genera with the meningococcal reference system are given in Table 1. Fig. 1, A–D shows a comparison between *Yersinia enterocolitica* antigens and meningococcal antigens.

Four antigens were found in a wide range of genera, mostly gram negative bacteria. Antigen no. 19 was found in 16 different genera in both gram positive and gram-negative bacteria, antigen no. 38 in 12 different genera in both gram-positive and gram negative bacteria, and antigens nos. 22 and 41 in 8 and 5 different genera of gram-negative bacteria, respectively.

Staphylococcus, *Streptococcus* and *Bacteroides* were the only genera which showed no cross-reactions with *N. meningitidis* antigens.

Other *Neisseria* species were found to cross-react very extensively with the 48 *N. meningitidis* antigens investigated (see Fig. 2), notably *N. subflava* which cross reacted with all *N. meningitidis* antigens.

Control experiments showed that the meningococcal Ref-ag could absorb all antibodies in the Ref-ab which reacted with antigens from the various other *Neisseria* species.

Extending the previously described cross-reactions between antigens of *P. aeruginosa* and *B. pertussis*, it was found that meningococcal antigen no. 19 corresponds to *P. aeruginosa* antigen no. 10 and *B. pertussis* antigen no. 11. A tentative correspondence of the other antigens is outlined below.

	Antigens numbers			
N. meningitidis	19	22	38	41
P. aeruginosa	10	17	24	35
B. pertussis	11		28	

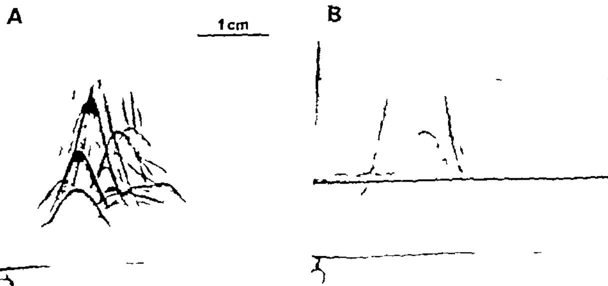


Fig. 2. Comparison of antigens from *Neisseria flavescens* with *Neisseria meningitidis* (Ref-ag) using a pooled rabbit antiserum against *N. meningitidis* (Ref-ab).
A. Crossed immunoelectrophoresis of *N. flavescens* against Ref-ab. Saline in the intermediate gel. The pattern resembles the reference pattern (Fig. 1, A).
B. Crossed-line immunoelectrophoresis of Ref-ag against Ref-ab with *N. flavescens* antigens in the intermediate gel (absorption of antibodies *in situ*). Of the precipitates which remain after absorption, some are unchanged, indicating that the corresponding antigens are not cross-reactive, and the areas of some precipitates are increased indicating partial identity between the corresponding antigens.

Acta path. microbiol. scand. Sect. B, 86: 93–100, 1978.

THE IMPORTANCE OF C5 AND THE ROLE OF THE ALTERNATIVE COMPLEMENT PATHWAY IN LEUKOCYTE CHEMOTAXIS INDUCED *IN VIVO* AND *IN VITRO* BY *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE

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Sveen, K. The importance of C5 and the role of the alternative complement pathway in leukocyte chemotaxis induced in vivo and in vitro by *Bacteroides fragilis* lipopolysaccharide. Acta path. microbiol. scand. Sect. B, 86: 93–100, 1978.

Chambers implanted subcutaneously in C5 normal (C5 N) and C5 deficient (C5 D) mice were used to examine the migration of polymorphonuclear leukocytes (PMNs) into the wound chamber fluid in response to injected *Bacteroides fragilis* lipopolysaccharide (LPS). The difference in PMN migration was highly significant between the two mouse strains, the C5 D mice showing no initial, but a low, delayed migration. The results from the study indicated that chemotaxis plays a major role in the accumulation of PMNs in the acute inflammatory response. Intraperitoneal endotoxin stimulation also showed a significantly lower total number of leukocytes in the exudate from C5 D mice as well as a delayed migration of cells compared to C5 N mice. No leukotactic mediators were elaborated in C5 D serum or exudate upon incubation with LPS when tested in a modified Boyden chamber. However, endotoxin-induced wound chamber fluid in C5 D mice showed an increasing leukotactic activity at the same time as the acute inflammatory response subsided in C5 N mice. Incubation of *B. fragilis* LPS in C4 deficient (C4 D) guinea pig serum indicated that the LPS was able to activate complement components to generate split products chemotactic for rabbit PMNs via the alternative complement pathway.

Key words: C5; alternative complement pathway; chemotaxis; *Bacteroides fragilis*; lipopolysaccharide.

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The endotoxicity of lipopolysaccharides (LPS) from *Bacteroides fragilis* has been questioned (7). Nevertheless, investigations carried out in this laboratory have shown that *B. fragilis* LPS produce primary skin inflammation as well as local and generalized Shwartzman reaction in rabbits (12). In addition, the LPS were lethal for mice and chick embryos, pyrogenic in rabbits and caused gelation of *Limulus* amoebocyte lysate (16). In all these tests the endotoxin activity was characteristically low. The *B. fragilis* LPS, which were chemotactically active *in vivo* (14), also showed a serum-dependent *in vitro* chemotaxis for polymorphonuclear leukocytes (PMNs) (13).

Snyderman et al. (10) have shown that complement (C) is implicated in the early accumulation of PMNs in inflammatory exudates produced by LPS. During the elaboration of these inflammatory mediators, which are cleavage products from C formed during the interaction with LPS, there is a pronounced consumption of the terminal C components C3 through C9, but only minimal using up of C1, C4 and C2 (4, 5, 6). The C5 was the main factor for mediation of the chemotactic activity (10, 11).

The present study was performed to examine the importance of various C components, particularly the C5, in the elaboration of leukotactic mediators upon interaction of *B. fragilis* LPS. Mice genetically

systemic *E coli* infections. Since the cross-reactive antibodies were present both in normal human serum (8) and in serum from patients early during meningococcal infection (7), these antibodies do not seem to be protective against meningococcal infections

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(Nuclepore Filter Corp.) were used to separate the chamber in two compartments. The bottom compartment, having a volume of 0.2 ml, contained the fluid to be tested for chemotactic activity. The ratio of the complement source to Gey's medium, with or without LPS, was 1:1. Aliquots of 1.2 ml of the mixtures or controls were preincubated in a water-bath at 37° C for 30 min and thereafter at 56° C for 30 min.

Exudate aspirated from in vivo chambers 5 and 20 h after injection of endotoxin was preincubated at 56° C only before being tested for chemotactic activity. The volume of 0.4 ml of the leukocyte suspension was spun down on the filter (58 × g for 1.5 min) in a cytocentrifuge (Shandon Elliot Cytospin SCA-0030), thus giving a total of approximately 4×10^6 PMNs on the upper surface (13) and the top compartment filled up with 0.4 ml of Gey's medium containing antibiotics and BSA. After 3 h of incubation in humid air at 37° C the filters were removed, stained and cleared. These were then mounted as described previously (13). Chemotactic activity was quantitated by counting the numbers of PMNs, which had migrated completely through the filters, in five high-power fields under light microscopy (13).

Statistical Methods

For evaluation of differences of chemotactic activities, the Wilcoxon rank test for two samples (two-tailed) was used (3). Values of $p > 0.05$ were not accepted as statistically significant. The Pearson correlation coefficient (3) was used for calculation of the linearity of the dose-response curves.

EXPERIMENTS AND RESULTS

Seven days after the implantation, the chambers were surrounded by a granulation tissue, buds of which were growing through the perforations of the chamber wall, thus forming a lining of its lumen. The survival at this time was eight out of ten C5 D and all ten of C5 N mice.

The volume of exudate aspirated from the chambers in the C5 N and C5 D mice seven days after implantation (time zero) and 5 h and 20 h after injection of 50 µg LPS-E 323 in 0.2 ml saline or saline alone into the chambers, is shown in Table 1.

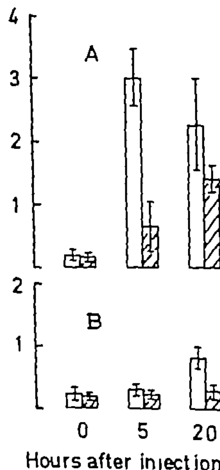


Fig. 1. Total number of leukocytes in wound chamber fluid seven days after implantation (0) and five and 20 h after application of 100 µg LPS-E 323 in 0.2 ml saline, or only saline, in C5 N (A) and C5 D (B) mice. Each column represents the mean number of cells per wound chamber ± standard deviation (vertical bar) from five chambers except at time zero where the number was ten. □ endotoxin induced wound fluid, ▨ saline induced wound fluid.

TABLE 1. *Volume (µl) of Exudate in the Wound Chambers of C5 Normal (C5 N) and C5 Deficient (C5 D) Mice Before (Time Zero) and five and 20 h After Injection of LPS E 323 in Saline, or Saline alone*

Mouse strain	Before injection	Five h after injection		20 h after injection	
	Mean ± s.d.	LPS Mean ± s.d.	Saline Mean ± s.d.	LPS Mean ± s.d.	Saline Mean ± s.d.
C5 N	156 32	141 36	147 ± 47	161 ± 46	150 ± 32
C5 D	179 ± 41	175 ± 77	198 65	116 ± 43	118 20

Mean and standard deviation (s.d.) are calculated from five chambers. The difference in amount of exudate aspirated from C5 N and C5 D mice was not significant either before or after the stimulation with LPS or saline.

deficient in C5 (C5 D) and C5 normal mice (C5 N), were employed in the experiments. Furthermore, the ability of *B. fragilis* LPS to activate the alternative complement pathway in the induction of chemotactic factor production was examined using guinea pig serum genetically deficient in C4 (C4 D).

MATERIALS AND METHODS

Source and Preparation of LPS

LPS (endotoxin) was isolated from *Bacteroides fragilis* ss. *fragilis* strain Lille E 323 by phenol water extraction (17) and purified by ultracentrifugation and treatment with ribonuclease and deoxyribonuclease (12). Stock suspensions of LPS were made in sterile isotonic saline (0.85 per cent NaCl) for *in vivo* assays and in Gey's balanced salt solution (Gey's medium) for *in vitro* assays and sonicated (MSE Mullard, 60w. 20 kc/s) for one min at 0° C. The pH was adjusted to 7.2 using triethylamine.

Animals

Mice of both sexes of the C5 normal strain NMRJ weighing 32 ± 3 g, and of the strain DBA/2J genetically deficient in C5 weighing 28 ± 2 g were used, the two mouse strains being caged separately. Adult guinea pigs, normal and genetically deficient in C4 (homozygous) (supplied by MRC Lab. Animals Centre, Surrey, England), were used. Their weights ranged from 400–600 g, and the two strains were housed separately. New Zealand White rabbits, 5–6-months-old and weighing 3.5–4.0 kg. were also caged separately. All animals were maintained on a standard laboratory diet and water *ad lib.* They were kept at a temperature of 20–22° C and a relative humidity of 45–50.

Measurement of Leukocyte Migration into Wound Chambers

Teflon® chambers (8) of 22 mm length and with a diameter of 6 mm, were implanted subcutaneously in the mice, one on each lateral side of the body of each animal. The preoperative preparation of the mice and the operative procedure was performed as described earlier (15). Seven days after the implantation, the formed exudate (wound fluid) in the chamber was aspirated, and the volume measured. The exudate was immediately brought to 0° C in an ice-bath, after which 25 µl of exudate were added to 475 µl of methylene blue, and the number of leukocytes counted using a Burker chamber. The aspirated exudate was replaced by a suspension of 50 µg of LPS-E 323 in 0.2 ml of sterile isotonic saline into one chamber and the same amount of saline into the other chamber as control. At different time intervals after the application of the test substances, the exudate was aspirated and its volume measured, and the number of leukocytes per wound chamber calculated. The exudate aspirated seven days after the implantation of the chambers was checked for contaminating microorganisms by microscopy of Gram stained preparations and from anaerobic and aerobic incubation on blood agar plates.

Measurement of Migration of Leukocytes into the Peritoneal Cavity

Suspensions of LPS-E 323 in amounts of 150 µg in 0.9 ml sterile isotonic saline were injected intraperitoneally (i.p.). At different time intervals thereafter the mice were sacrificed by cervical dislocation, and the peritoneal cavity was exposed by an abdominal incision. The exudate was collected either by rinsing the peritoneal cavity with 0.005 M sodium ethylenediamine tetraacetate (EDTA) in saline (pH 7.2) to block the activation of complement (10) or only saline, both containing 10 I.U. of heparin (A/S Apothekernes Laboratorium for Specialpreparater. Oslo) per ml and immediately brought to 0° C. The volume of exudate from each mouse was adjusted to a volume of 2.7 ml, and 25 µl of this transferred to 475 µl of methylene blue to determine the number of leukocytes per µl of exudate.

Chemotactic Assays

Polymorphonuclear leukocytes. Polymorphonuclear leukocytes (PMNs) were harvested from the peritoneal cavity of rabbits 8–10 h after i.p. stimulation with 200 ml of a solution containing 0.1 per cent glycogen (E. Merck AG. Darmstadt, W.-Germany) in sterile isotonic saline. After isolation of the PMNs by centrifugation at 225 × g for 5 min (IEC Universal Model UV), they were resuspended in Gey's medium containing 50 µg garamycin® (Schering Corporation, Bloomfield. N.J. USA) and 2.5 µg fungizone® (Squibb Flow Laboratories, Irvine, Scotland) per ml and 2 per cent bovine serum albumin (BSA) (Armour Pharmaceutical Company Ltd., Eastbourne, England). The leukocyte suspension was adjusted to give a final number of approximately 10^7 leukocytes per ml. The media used were sterilized before use by passage through a filter with a pore size of 0.45 µ (Millipore Filter Corp. Bedford, Mass. USA).

Complement Source

Fresh, pooled serum from genetically C4 D and C4 N guinea pigs, as well as from C5 D and C5 N mice, was stored at −25° C in aliquots of one ml. In addition, exudate aspirated from the implanted chambers before and after stimulation with LPS was used. The exudate was centrifuged at 28 000 × g for 20 min at 4° C (Servall RC 2. Sorvall Inc., Norwalk, Conn. USA) and the supernatant fluid frozen in aliquots of one ml. Endotoxin or saline stimulated peritoneal exudate was centrifuged at 28 000 × g and lyophilized. The lyophilized exudate from three mice was redissolved in 1 ml of distilled water and dialyzed against saline of at least 1000 times their volume using a magnetic mixer at 4° C for 24 h and thereafter lyophilized. Before testing on chemotactic activity the freeze-dried exudate was dissolved in 0.8 ml of Gey's medium, thus giving a concentration of the exudate ten times higher than at the harvesting.

Measurement of Chemotactic Activity

Chemotaxis of rabbit PMNs was quantitatively assayed by the Boyden Micropore filter technique (1) using a modified Boyden chamber (11) (Neuroprobe, Bethesda, Md. USA). Filters of 3.0 µ pore size

(Millipore Filter Corp) were used to separate the chamber in two compartments. The bottom compartment, having a volume of 0.2 ml, contained the fluid to be tested for chemotactic activity The ratio of the complement source to Gey's medium, with or without LPS, was 1:1 Aliquots of 1 2 ml of the mixtures or controls were preincubated in a water-bath at 37° C for 30 min and thereafter at 56° C for 30 min

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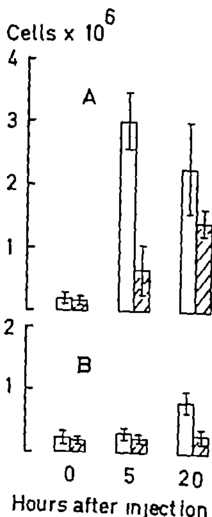


Fig 1 Total number of leukocytes in wound chamber fluid seven days after implantation (0) and five and 20 h after application of 100 μg LPS-E 323 in 0 2 ml saline, or only saline in C5 N (A) and C5 D (B) mice Each column represents the mean number of cells per wound chamber ± standard deviation (vertical bar) from five chambers except at time zero where the number was ten ☐ endotoxin induced wound fluid, ▨ saline induced wound fluid

TABLE 1 *Volume (μl) of Exudate in the Wound Chambers of C5 Normal (C5 N) and C5 Deficient (C5 D) Mice Before (Time Zero) and five and 20 h After Injection of LPS-E 3 3 in Saline, or Saline alone*

Mouse strain	Before injection	Five h after injection		20 h after injection	
	Mean ± s d	LPS Mean ± s d	Saline Mean ± s d	LPS Mean ± s d	Saline Mean ± s d.
C5 N	156 32	141 36	147 ± 67	161 ± 46	150 ± 32
C5 D	179 ± 41	175 ± 77	198 65	116 43	118 ± 20

Mean and standard deviation (s d) are calculated from five chambers The difference in amount of exudate aspirated from C5 N and C5 D mice was not significant either before or after the stimulation with LPS or saline

deficient in C5 (C5 D) and C5 normal mice (C5 N), were employed in the experiments. Furthermore, the ability of *B fragilis* LPS to activate the alternative complement pathway in the induction of chemotactic factor production was examined using guinea pig serum genetically deficient in C4 (C4 D).

MATERIALS AND METHODS

Source and Preparation of LPS

LPS (endotoxin) was isolated from *Bacteroides fragilis* ss. *fragilis* strain Lille E 323 by phenol water extraction (17) and purified by ultracentrifugation and treatment with ribonuclease and deoxyribonuclease (12). Stock suspensions of LPS were made in sterile isotonic saline (0 85 per cent NaCl) for *in vivo* assays and in Gey's balanced salt solution (Gey's medium) for *in vitro* assays and sonicated (MSE Mullard, 60w 20 kc/s) for one min at 0° C. The pH was adjusted to 7 2 using triethylamine.

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Mice of both sexes of the C5 normal strain NMRJ weighing 32 ± 3 g and of the strain DBA/2J genetically deficient in C5 weighing 28 ± 2 g were used the two mouse strains being caged separately Adult guinea pigs, normal and genetically deficient in C4 (homozygous) (supplied by MRC Lab Animals Centre, Surrey England) were used. Their weights ranged from 400–600 g, and the two strains were housed separately New Zealand White rabbits, 5–6-months-old and weighing 3 5–4 0 kg, were also caged separately All animals were maintained on a standard laboratory diet and water *ad lib* They were kept at a temperature of 20–22° C and a relative humidity of 45–50

Measurement of Leukocyte Migration into Wound Chambers

Teflon® chambers (8) of 22 mm length and with a diameter of 6 mm, were implanted subcutaneously in the mice, one on each lateral side of the body of each animal. The preoperative preparation of the mice and the operative procedure was performed as described earlier (15) Seven days after the implantation, the formed exudate (wound fluid) in the chamber was aspirated, and the volume measured. The exudate was immediately brought to 0° C in an ice-bath, after which 25 µl of exudate were added to 475 µl of methylene blue, and the number of leukocytes counted using a Burker chamber The aspirated exudate was replaced by a suspension of 50 µg of LPS-E 323 in 0 2 ml of sterile isotonic saline into one chamber and the same amount of saline into the other chamber as control At different time intervals after the application of the test substances, the exudate was aspirated and its volume measured, and the number of leukocytes per wound chamber calculated. The exudate aspirated seven days after the implantation of the chambers was checked for contaminating microorganisms by microscopy of Gram stained preparations and from anaerobic and aerobic incubation on blood agar plates.

Measurement of Migration of Leukocytes into the Peritoneal Cavity

Suspensions of LPS-E 323 in amounts of 150 µg in 0 9 ml sterile isotonic saline were injected intraperitoneally (i.p.). At different time intervals thereafter the mice were sacrificed by cervical dislocation, and the peritoneal cavity was exposed by an abdominal incision. The exudate was collected either by rinsing the peritoneal cavity with 0 005 M sodium ethylenediamine tetraacetate (EDTA) in saline (pH 7 2) to block the activation of complement (10) or only saline, both containing 10 I.U of heparin (A/S Apothekernes Laboratorium for Speci alpræparater Oslo) per ml and immediately brought to 0° C. The volume of exudate from each mouse was adjusted to a volume of 2.7 ml, and 25 µl of this transferred to 475 µl of methylene blue to determine the number of leukocytes per µl of exudate.

Chemotactic Assays

Polymorphonuclear leukocytes. Polymorphonuclear leukocytes (PMNs) were harvested from the peritoneal cavity of rabbits 8–10 h after i p. stimulation with 200 ml of a solution containing 0 1 per cent glycogen (E Merck AG Darmstadt, W-Germany) in sterile isotonic saline. After isolation of the PMNs by centrifugation at 225 × g for 5 min (IEC, Universal Model UV) they were resuspended in Gey's medium containing 50 µg garamycin® (Schering Corporation, Bloomfield, N J USA) and 2.5 µg fungizone® (Squibb Flow Laboratories, Irvine, Scotland) per ml and 2 per cent bovine serum albumin (BSA) (Armour Pharmaceutical Company Ltd Eastbourne, England). The leukocyte suspension was adjusted to give a final number of approximately 10^7 leukocytes per ml The media used were sterilized before use by passage through a filter with a pore size of 0 45 µ (Millipore Filter Corp Bedford, Mass USA)

Complement Source

Fresh, pooled serum from genetically C4 D and C4 N guinea pigs, as well as from C5 D and C5 N mice, was stored at −25° C in aliquots of one ml In addition, exudate aspirated from the implanted chambers before and after stimulation with LPS was used The exudate was centrifuged at 28 000 × g for 20 min at 4 C (Sorvall RC-2, Sorvall Inc., Norwalk, Conn USA) and the supernatant fluid frozen in aliquots of one ml Endotoxin or saline stimulated peritoneal exudate was centrifuged at 28 000 × g and lyophilized The lyophilized exudate from three mice was redissolved in 1 ml of distilled water and dialyzed against saline of at least 1000 times their volume using a magnetic mixer at 4° C for 24 h and thereafter lyophilized. Before testing on chemotactic activity the freeze-dried exudate was dissolved in 0 8 ml of Gey's medium, thus giving a concentration of the exudate ten times higher than at the harvesting

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Chemotaxis of rabbit PMNs was quantitatively assayed by the Boyden Micropore filter technique (1) using a modified Boyden chamber (11) (Neuroprobe, Bethesda, Md USA) Filters of 3 0 µ pore size

(Millipore Filter Corp.) were used to separate the chamber in two compartments. The bottom compartment, having a volume of 0.2 ml, contained the fluid to be tested for chemotactic activity. The ratio of the complement source to Gey's medium, with or without LPS, was 1:1. Aliquots of 1.2 ml of the mixtures or controls were preincubated in a water-bath at 37° C for 30 min and thereafter at 56° C for 30 min.

Exudate aspirated from *in vivo* chambers 5 and 20 h after injection of endotoxin was preincubated at 56° C only before being tested for chemotactic activity. The volume of 0.4 ml of the leukocyte suspension was spun down on the filter (58 × g for 1.5 min) in a cytocentrifuge (Shandon Elliot Cytospin SCA-0030), thus giving a total of approximately 4×10^6 PMNs on the upper surface (13) and the top compartment filled up with 0.4 ml of Gey's medium containing antibiotics and BSA. After 3 h of incubation in humid air at 37° C the filters were removed, stained and cleared. These were then mounted as described previously (13). Chemotactic activity was quantitated by counting the numbers of PMNs, which had migrated completely through the filters, in five high-power fields under light microscopy (13).

Statistical Methods

For evaluation of differences of chemotactic activities, the Wilcoxon rank test for two samples (two-tailed) was used (3). Values of $p > 0.05$ were not accepted as statistically significant. The Pearson correlation coefficient (3) was used for calculation of the linearity of the time-response curves.

EXPERIMENTS AND RESULTS

Seven days after the implantation, the chambers were surrounded by a granulation tissue, buds of which were growing through the perforations of the chamber wall, thus forming a lining of its lumen. The survival at this time was eigth out of ten C5 D and all ten of C5 N mice.

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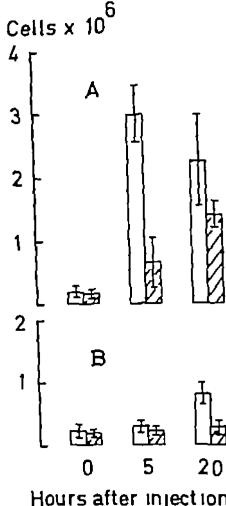


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Mouse strain	Before injection	Five h after injection		20 h after injection	
		LPS	Saline	LPS	Saline
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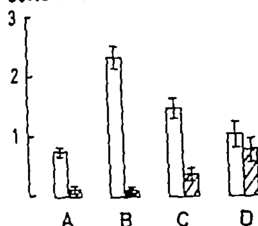


Fig 3 Exudate aspirated from chambers implanted in mice and tested for chemotaxis in a modified Boyden chamber. Each column represents the mean number of cells on the chemotactic side of the filter disc ± standard deviation (vertical bar) from five filters. Chemotaxis induced by exudate aspirated seven days after implantation without LPS (A) and incubated with 100 µg LPS-E 323 (B). C and D represent chemotaxis induced by inflammatory exudate aspirated five and 20 h after local LPS injection, respectively. □ C5 N exudate, ▨ C5 D exudate

mice. The cell accumulation in C5 D mice was not distinctly above the control counts until seven h after the endotoxin was instilled i.p. A rather steep ascending time-response relationship in C5 N mice compared with that of C5 D mice was found. However, a significant correlation was found between the time after endotoxin injection and accumulation of cells both in C5 N ($r = 0.99$, $p < 0.001$) and in C5 D ($r = 0.92$, $p < 0.005$) mice. The peak in peritoneal cell accumulation in C5 D mice at 20 h was only 25 per cent of that in C5 N mice. The time-response curves (standard deviations of the means) after endotoxin injection did not overlap each other at any time interval. In the time interval of seven to 20 h, however, the saline induced time-response curves overlapped each other.

An *in vitro* experiment for measurement of chemotaxis from exudates aspirated from wound chambers of C5 N and C5 D mice at time zero and five and 20 h after the application of endotoxin is shown in Fig. 3. Exudates aspirated from C5 D mice seven days after implantation rendered a non-stimulated activity of about 15 per cent only of that from C5 N mice. Incubation of the same exudates with 100 µg of LPS-E 323 per 0.1 ml exudate indicated that no chemotactic mediators were generated from C5 D exudate giving cell counts even lower than the controls. The exudates aspirated five h after endotoxin application were found chemotactically active in both mouse strains, but the exudate from C5 N mice rendered about four times higher cell counts than that from C5 D mice ($p < 0.01$). At 20 h however, no significant difference in chemotactic activity between the exudates was found ($p > 0.05$).

The capacity of endotoxin to induce chemotaxis when incubated in serum from C5 N and C5 D mice is shown in Fig. 4. No leukotactic mediators were elaborated from serum deficient in C5; the difference in chemotactic activity to serum alone was not statistically significant ($p > 0.05$). Even the cell counts from C5 N serum was higher than the number of cells per high-power field from the C5 D serum-endotoxin mixture ($p < 0.01$).

The chemotactic activity exerted on rabbit PMNs by C4 N and C4 D guinea pig serum upon

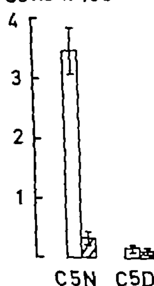


Fig 4 Leukocyte chemotaxis from mouse serum upon incubation with 100 µg LPS-E 323 and tested in a modified Boyden chamber. Each column represents the mean number of cells per high-power field on the chemotactic side of the filter ± standard deviation (vertical bar) from five filters. C5 N = C5 normal serum, C5 D = C5 deficient serum. □ serum + LPS, ▨ serum alone.

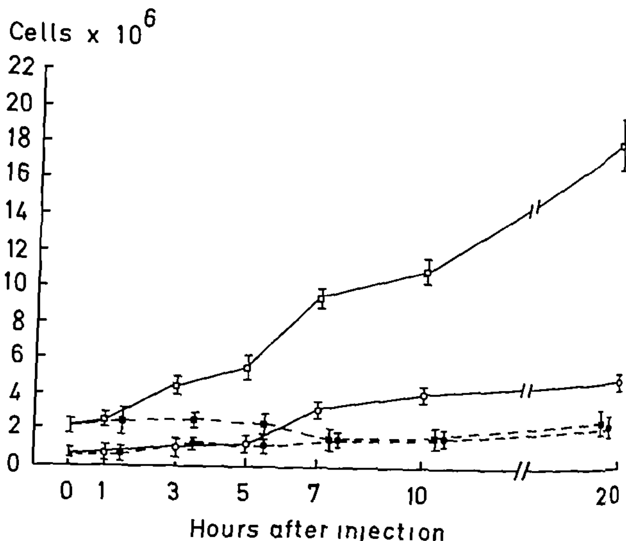


Fig. 2 Kinetics of leukocyte accumulation in the peritoneal cavity of C5 N and C5 D mice after local injection of 50 µg LPS-E 323 in 0 9 ml saline, or saline alone. Each point represents the mean cell count standard deviation (vertical bar) from three mice. There is a time difference of half an hour between the aspiration of endotoxin and saline induced exudate.
-□- C5 N mice injected with LPS -○- C5 D mice injected with LPS
-■- C5 N mice injected with saline -●- C5 D mice injected with saline

Comparison of the amount of exudate from chambers injected with endotoxin suspension to that from chambers containing saline 5 h and 20 h later gave no statistical significance in either of the mouse strains ($p > 0.05$)

At time zero no significant difference in the total number of cells per wound chamber was to be observed when C5 N and C5 D mice were compared ($p > 0.05$) (Fig. 1) Five h after the application of endotoxin the total number of cells in the wound chamber fluid from C5 N mice was significantly higher than that from C5 D mice ($p < 0.01$). The control chambers of C5 N mice contained wound fluid more rich in cells than the corresponding chambers of C5 D mice ($p < 0.01$) When comparing the total content of cells in chambers inoculated with endotoxin to those containing saline, the higher number of cells in endotoxin induced wound fluid was statistically significant in C5 N mice ($p < 0.01$) but there was no difference in C5 D mice ($p > 0.05$). Twenty h after injection of endotoxin, or saline only the higher total number of cells in chambers of C5 N than of C5 D mice, was at the level of one per cent both in endotoxin and in saline induced wound fluid. At this time the number of cells accumulated in the endotoxin induced exudate was similar to that of the control exudate in C5 N mice, but the endotoxin induced wound chamber fluid contained a significantly higher number of cells than that induced by saline in C5 D mice ($p < 0.01$).

Kinetics of leukocyte accumulation intraperitoneally after stimulation with endotoxin in the C5 N were quite different from that of C5 D mice (Fig. 2) Three h after injection of endotoxin a significant increase in the influx of cells was found in C5 N

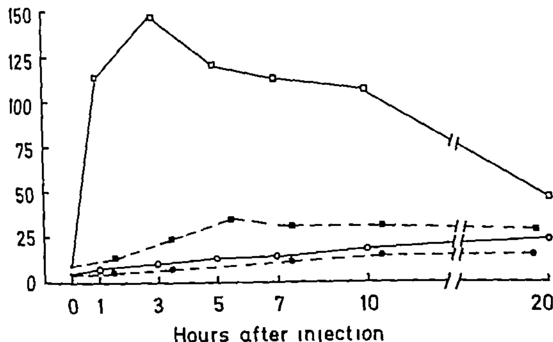


Fig 4 Leukocyte chemotaxis from the same peritoneal exudate as shown in Fig 2, tested in a modified Boyden chamber device. Each point represents the mean number of cells on the chemotactic side of the filter from two filters. –□– endotoxin stimulated exudate in C5 N mice, –■– saline stimulated exudate in C5 N mice, –O– endotoxin stimulated exudate in C5 D mice, –●– saline stimulated exudate in C5 D mice

was injected intraperitoneally. This finding corroborates that of *Snyderman et al* (10) who also demonstrated a peak in mediator concentration of the exudate in the early phase of the inflammatory response after local injection of endotoxin.

Problems were encountered in the experiments using C5 D mice as they easily succumbed to infection, which also has been experienced by others (2, 9). The delay in accumulation of PMNs in C5 D mice in *in vivo* chamber as well as in the peritoneal cavity after local endotoxin injection may therefore suggest this gap in the mechanism of defence to be a contributing factor to their relatively high mortality rate.

The *B fragilis* LPS prompted an escape of fluid into the wound chamber of both C5 N and C5 D mice. A decrease in volume of wound chamber fluid was found to parallel the increased number of cells after local endotoxin stimulation in C5 D mice, whereas an increase in volume of wound chamber fluid, but a decrease in cell number accumulation in C5 N mice was found in the late phase of the inflammatory response. Though there normally may be an increased permeability of the vessels of the granulation tissue within the chamber in connection with the application of LPS to normal mice, these results indicate that a vascular dilatation may play a minor role in the accumulation of PMNs in an acute inflammatory response.

REFERENCES

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incubation with endotoxin is demonstrated in Fig 5 The migration induced by cytotaxins generated in C4 D serum, was almost of the same order of magnitude as the migration induced by cytotaxins generated in C4 N serum by *B fragilis* LPS. The difference in chemotactic stimulation provided by the two sera was not statistically significant ($p > 0.05$).

In vitro tests on the chemotactic activity exerted on rabbit PMNs from pooled peritoneal exudate of C5 N mice concentrated ten times, was most pronounced 3 h after the local injection of LPS (Fig. 6). In contrast, the chemotactic activity from the supernatants of the exudate from C5 D mice was not found before 20 h and was very low The quantitative cell responses of exudate harvested from the peritoneal cavity using EDTA or saline were similar

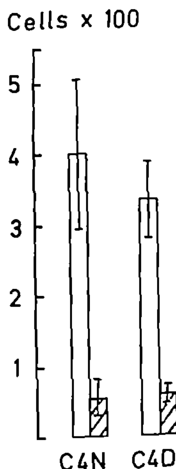


Fig 5 Leukocyte chemotaxis from LPS-E 323 in doses of 100 µg upon incubation with C4 normal (C4 N) and C4 deficient (C4 D) guinea pig serum and tested in a modified Boyden chamber Each column represents the mean number of cells per high-power field on the chemotactic side of the filter ± standard deviation (vertical bar) from five filters. □ serum + LPS ▨ serum alone.

DISCUSSION

The main implications of the present study are that LPS isolated from *B fragilis* promote a C5 dependent leukocyte chemotaxis in mice, and that the leukocyte chemotaxis *in vitro* induced by the same LPS takes place in the absence of C4 but not C5 Therefore, this shows that *B fragilis* LPS like LPS from *Veillonella* (11) and *Salmonella* (10), induces the production of chemotactic mediators via the alternative complement pathway

The data from the *in vivo* experiments as well as from the *in vitro* studies, show that C5 D mice are unable to generate mediators chemotactic for PMNs in the early phase of the inflammatory response to *B fragilis* LPS These findings and results obtained by others (10 11) show that chemotactic factors essential in the inflammatory response, are products from C components, and that the main chemotactic mediator probably is a split product from C5 The results of this study also corroborate the findings of *Frank et al.* (5) that endotoxins activate the late complement factors (C3–C9) via the alternative pathway the reason being no significant difference in chemotactic factor elaboration upon incubation with C4 N and C4 D guinea pig serum (cf Fig 5) This implies that the chemotaxis measured is not the result of an interaction between antibodies in the serum or exudate with the *B fragilis* LPS.

The accumulation of PMNs in the wound chamber fluid of the C5 D mice was not marked until 20 h after the local injection of LPS (cf Fig 1) The indication of an inflammatory defence mechanism functioning in C5 D mice could also be demonstrated in *in vitro* examination of the endotoxin stimulated exudate. Thus, there was detectable chemotactic activity of the wound chamber fluid aspirated five h after the injection of LPS into the chamber and a marked chemotaxis of that after 20 h (cf Fig 3). These findings do not give conclusive evidence as to the nature of this late attraction activity However from the results presented, chemotaxis seemed to be responsible for the leukocyte migration *in vivo*

The late intraperitoneal cell accumulation after local stimulation with LPS-E 323 in C5 D mice (cf Fig 2) is in agreement with the findings of *Snyderman et al* (10). At any time interval, the response, expressed as the accumulation of PMNs, was two to four times higher in the C5 N mice Thus, the pattern of their time-response curves was quite similar as shown by their respective correlation coefficients. Examination of chemotactic activity of peritoneal exudate from C5 N mice *in vitro* demonstrated a peak in mediator concentration corresponding to that harvested 3 h after endotoxin

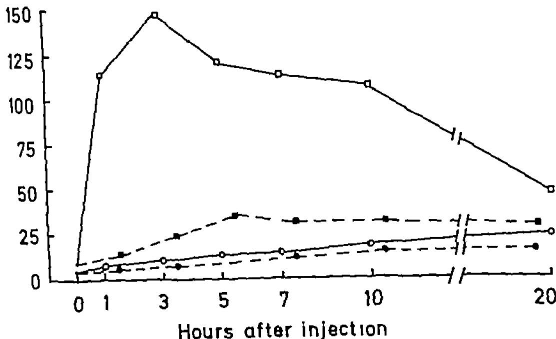


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incubation with endotoxin is demonstrated in Fig 5 The migration induced by cytotaxins generated in C4 D serum, was almost of the same order of magnitude as the migration induced by cytotaxins generated in C4 N serum by *B fragilis* LPS The difference in chemotactic stimulation provided by the two sera was not statistically significant ($p > 0.05$).

In vitro tests on the chemotactic activity exerted on rabbit PMNs from pooled peritoneal exudate of C5 N mice concentrated ten times, was most pronounced 3 h after the local injection of LPS (Fig 6). In contrast, the chemotactic activity from the supernatants of the exudate from C5 D mice was not found before 20 h and was very low The quantitative cell responses of exudate harvested from the peritoneal cavity using EDTA or saline were similar

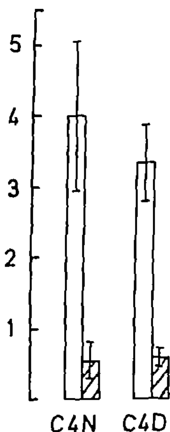


Fig 5 Leukocyte chemotaxis from LPS-E 323 in doses of 100 µg upon incubation with C4 normal (C4 N) and C4 deficient (C4 D) guinea pig serum and tested in a modified Boyden chamber Each column represents the mean number of cells per high-power field on the chemotactic side of the filter ± standard deviation (vertical bar) from five filters. □ serum + *LPS* ▨ *serum alone*.

DISCUSSION

The main implications of the present study are that LPS isolated from *B fragilis promote a C5* dependent leukocyte chemotaxis in mice, and that the leukocyte chemotaxis *in vitro* induced by the same LPS takes place in the absence of C4 but not C5 Therefore, this shows that *B fragilis* LPS, like LPS *from Veillonella* (11) and *Salmonella* (10) induces the production of chemotactic mediators via the alternative complement pathway

The data from the *in vivo* experiments as well as from the *in vitro* studies, show that C5 D mice are unable to generate mediators chemotactic for PMNs in the early phase of the inflammatory response to *B fragilis* LPS These findings and results obtained by others (10 11), show that chemotactic factors essential in the inflammatory response, are products from C components, and that the main chemotactic mediator probably is a split product from C5 The results of this study also corroborate the findings of *Frank et al.* (5) *that endotoxins activate the late* complement factors (C3–C9) via the alternative pathway the reason being no significant difference in chemotactic factor elaboration upon incubation with C4 N and C4 D guinea pig serum (cf Fig 5) *This implies that the chemotaxis measured is not* the result of an interaction between antibodies in the serum or exudate with the *B fragilis* LPS.

The accumulation of PMNs in the wound chamber fluid of the C5 D mice was not marked until *20 h* after the local injection *of LPS* (cf Fig. 1) The indication of an inflammatory defence mechanism functioning in C5 D mice could also be *demonstrated in in vitro* examination of the endotoxin stimulated exudate. Thus, there was detectable chemotactic activity of the wound cham ber fluid asperated five h after the injection of LPS into the chamber and a marked chemotaxis of that *after 20 h* (*cf Fig* 3). *These findings* do not give conclusive evidence as to the nature of this late attraction activity However from the results presented, chemotaxis seemed to be responsible for the leukocyte migration *in vivo*

The late *intraperitoneal cell* accumulation after local stimulation with LPS-E 323 in C5 D mice (cf Fig. 2) is in agreement with the findings of *Snyderman et al.* (10). At any time interval, the response, expressed as the accumulation of PMNs, was two to *four times higher* in the C5 N mice Thus, the pattern of their time-response curves was quite similar as shown by their respective correla tion coefficients. Examination of chemotactic acti vity of peritoneal exudate from C5 N mice *in vitro demonstrated a peak* in mediator concentration corresponding to that harvested 3 h after endotoxin

Acta path microbiol scand Sect B, 86 101–106 1978

BACTERIAL CONTAMINATION OF HEAT-STERILIZED, HEAT DISINFECTED AND CHEMICALLY DISINFECTED HAEMODIALYSIS MONITORS

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Kolmos, H J Bacterial contamination of heat-sterilized, heat-disinfected and chemically-disinfected haemodialysis monitors Acta path microbiol scand Sect B, 86 101–106 1978

The bacterial contamination of one heat-sterilized, three heat-disinfected and four chemically-disinfected monitor types was evaluated before and after dialysis All monitor types were contaminated In the heat-treated monitors the level of contamination varied with the intensity of the heat treatment and the technical design. They were less contaminated than the chemically-disinfected recirculating single-pass monitors The latter were contaminated to a marginal degree, irrespective of the quality of the water supply Each monitor showed a characteristic microbial flora, indicating that recontamination occurred from the same persistent focus Acquired antibiotic resistance characters were rare among the potential human pathogens isolated from the dialysate

The level of contamination during dialysis is determined by a complex of factors, including the mode of disinfection, the technical design of the dialysis equipment, the duration of the dialyses, and the flora of the dialysate

Key words Bacterial contamination, haemodialysis monitors, bacterial flora, antibiotic resistance

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Microbial contamination of the dialysate fluid used in haemodialysis represents a major hygienic problem Bacteria and their waste products may cross dialysis membranes (1 10) and cause pyrogenic reactions in the patients (4 7 9) and the proliferation of microorganisms may alter the chemical composition of the dialysate, resulting in dialysis acidosis (17). The dialysis fluid contains salts and organic waste products dialysed from the patient, during dialysis it is heated to body temperature, and thus it forms a good medium for a rapid microbial proliferation. Further the design of the dialysis equipment often favours microbial growth, the water supply system and the tubes leading the dialysis fluid to the monitors are easily colonized, especially at sites where the tubes are connected and where the flow of the dialysate is low (2, 19) Chemical disinfection is to a great extent inefficient (19). Within recent years promising results have been obtained with heat decontamination (5 18)

From 1974 to 1976 four new monitor types were tested in the haemodialysis centre at Odense University Hospital. Three of these could be disinfected by means of heat and one sterilized by autoclaving at 120°C for 40 minutes. This study describes the microbial contamination of these monitors, in comparison with four older chemically-disinfected monitor types, their microbial flora, and the degree of antibiotic resistance among the strains isolated

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TABLE 1 *Number of Microorganisms Isolated from the Monitors at the Start and End of the Dialyses*

Monitor type	T	N	log number of microorganisms					
			Start of dialysis			End of dialysis		
			mean	SD	range	mean	SD	range
A	6½	19	3.40	1.76	0–5.59	5.27	0.87	3.30–6.50
B	6	12	3.89	0.69	3.06–4.83	4.89	0.86	3.89–6.45
C	5½	32	2.05	0.77	0.70–4.55	3.13	0.84	1.69–6.02
D	5½	18	0.62	0.74	0–2.08	2.03	1.57	0–5.21
E	4½	27	0.66	0.82	0–2.60	2.34	1.65	0–5.74
F	5½	15	1.83	0.55	0.93–2.58	1.82	0.39	1.15–2.38
G	5½	13	0.55	0.56	0–1.53	0.44	0.52	0–1.88
H	5½	21	–	–	–	0.61	1.05	0–3.60

T mean duration of the dialyses (hours)
N number of observations
SD standard deviation
– not examined

view of the short duration of the dialyses. The bacterial findings after several heat disinfections of this monitor type are shown in Table 2. Monitor F (the monitor with the weakest heat treatment programme) was significantly contaminated at the start of dialysis, but the concentration of microorganisms did not increase during dialysis. The same was true of monitor G.

TABLE 2 *Results of 27 Heat Disinfections Performed with Monitor E*

	Number of specimens	Concentration/ 100 ml
No growth	23	
Bacillus	2	1
Micrococcus	1	>100
Yeasts	1	1

The recirculating single-pass monitors (A–C, disinfected with chloramine) were the most contaminated of all monitors tested, both before and after dialysis. In monitor A a negative linear correlation was registered between the number of microorganisms present originally and the increase during dialysis (Fig. 2). A similar correlation, though less marked, was observed in monitor B. The contamination of monitor D (disinfected with formaline) was within the range of the results obtained with the heat-treated monitors.

Table 3 shows the microorganisms isolated from the monitors at the end of the dialyses. Repeated cultures revealed a characteristic flora for each monitor type.

Table 4 shows a biochemical characterization of *Enterobacteriaceae* isolated more than once at the end of dialysis. The isolated strains of *E. coli* and *C. freundii* belonged to the same biotypes. Strains of *K. pneumoniae* were identical, except for the citrate

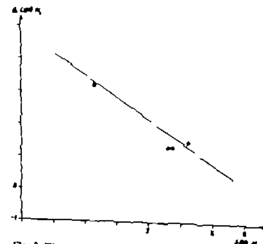


Fig. 2 The correlation between the number of microorganisms present at the start of dialysis and their increase during dialysis in monitor A.
Signature
N number of microorganisms / 0.1 ml at the start of dialysis
Δ LOG N the increase during dialysis
○ dialyses lasting 5 or 6 hours
● dialyses lasting 7 or 8 hours
$r = -0.87$ $y = -0.68x + 4.18$

MATERIAL AND METHODS

The dialysis centre at Odense University Hospital consists of a main unit for centre dialysis (monitors A, B D E, F G and H) and a separate training unit for home dialysis patients (monitor C).

The dialysis fluid used in the main unit was sterile, delivered in 200 litre plastic bags, either ready-mixed or as distilled water which was mixed with a concentrated salt solution in the monitors. From the plastic bags it was led to the monitors by a tube system, each monitor being supplied separately In the training unit the water supply was deionized tap water

Monitors A (Travenol RSP) B (Dasco) and C (Travenol CC) were of the recirculating single-pass type (15), in which the dialysate was recirculated through a coil dialyser and successively exchanged during dialysis. Monitors D (The Danish Sugar Factories) F (Gambro), G (Dylade), and H (Nycotron) were of the single-pass type (15), in which the dialysate was pumped through a plate or capillary cartridge kidney and exhausted after a single pass. Monitor E (Rhodial) contained a fixed dialysate volume of 75 litres, which was recirculated through a plate dialyser All dialysers were disposable units. The temperature of the dialysate was 38°C. The duration of the dialyses is shown in Table 1

Disinfection after dialysis took place as follows: monitors A, B and C were flushed with 1% chloramine for 10 minutes and stored in this solution between the dialyses. Before the next dialysis they were flushed again for 5 minutes and then rinsed with tap water Monitor D was disinfected with 2% formalin for 15 minutes and stored in this until the next dialysis. Before the start of dialysis it was rinsed with tap water Monitors E, F G and H were heat treated using demineralised water (monitor E: 92°C for 20 minutes, monitor F 85°C for 20 minutes, monitor G 85°C for 25 minutes both before and after dialysis, monitor H. autoclaving at 120°C for 40 minutes, the tubes leading the dialysate to and from the dialyser were stored in 1% phenoxalyl between the dialyses) In monitors G and H the dialysis fluid was heated temporarily to 80°C when entering the monitors

Samples of dialysis fluid were collected at the start and end of dialysis treatment from the monitors described above. Monitor H was only examined at the end of dialysis as it proved technically difficult to collect specimens before dialysis Monitor E was also examined after heat disinfection All samples were post membrane dialysate In the recirculating single-pass monitors they were collected from the top of the coil dialyser and in the single-pass monitors sampling was from the tube leading from the dialyser The concentration of dialysate microorganisms was determined from colony counts on 5% blood agar plates inoculated with 0 1 ml from a ten-fold series dilution in saline Viable counts after heat treatment were determined from a volume of 100 ml, using membrane filters which were placed on 5% blood agar plates The plates were left at room temperature for 72 hours before counting, and representative colonies were picked for identification (3 8 13 14 16) Biotyping of strains of *Klebsiella pneumoniae* was performed as described by *Ørskov* (20) Strains of *Citrobacter freundii Escherichia coli* and *Enterobacter cloacae* were subdivided according to the biochemical reactions given by *Edwards & Ewing* (6). They are shown in Table 3

Antibiotic sensitivity tests were carried out for all strains isolated, using the tablet method (Neosensitabs® Rosco, Taastrup Denmark) as described earlier (1)) Resistant strains had inhibition zone diametres corresponding to the following MIC's (ug/ml): sulphonamide ≥ 15 tetracyclines ≥ 4 chloramphenicol ≥ 15, streptomycin ≥ 20 gentamycin ≥ 11 polymyxines ≥ 20 ampicillin ≥ 5 and carbenicillin ≥ 10

RESULTS

The microbial contamination of the monitors is shown in Table 1 Microorganisms could be isolated from all monitor types tested at the start of dialysis. The heat treated monitors (E–H) showed a wide range of contamination. monitors G and H (those with the most intensive heat-treatment programmes) had the lowest degree of contamination of all monitors tested. In monitor H the degree of contamination increased during the course of the observation period (Fig. 1) – a pattern unique for this monitor type. Monitor E showed a relatively large increase in contamination during dialysis in

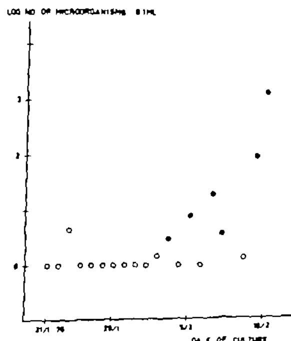


Fig 1 The concentration of microorganisms at the end of the dialyses in 21 consecutive cultures from monitor H

Signature:

● cultures from which *Enterobacteriaceae* were isolated.

O the remaining cultures

TABLE 5. *Individual Antibiotic Resistance Traits among Gramnegative Rods Isolated at the End of the Dialyses*

	N	Number of strains resistant							
		Su	Te	Ch	Po	St	Ge	Am	Ca
Escherichia coli	9	0	0	0	0	0	0	9	9
Klebsiella pneumoniae	18	17	0	0	0	1	0	18	18
Klebsiella oxytoca	1	0	0	0	0	0	0	1	1
Citrobacter freundii	12	0	0	1	0	0	0	12	10
Enterobacter cloacae	5	0	0	0	0	0	0	5	0
P. aeruginosa	60	46	1	4	0	9	0	60	11
P. putida (biotype 11)	20	19	0	16	0	14	0	20	20
Fluorescent *Pseudomonas* biotype 6	46	34	0	32	0	46	0	46	46
Other fluorescent Pseudomonads	5	2	0	3	0	0	0	5	5
P. maltophilia	15	1	11	0	0	15	15	15	15
Other Pseudomonads	31	7	2	0	3	5	2	25	22
Alcaligenes	11	0	0	10	0	11	11	9	9
Aeromonas formicans	12	0	0	0	0	0	0	12	10
Xanthomonas	2	0	0	0	2	2	0	2	2
A. calcoaceticus	5	0	0	0	0	0	0	0	0
Flavobacterium	2	0	0	0	2	0	2	2	2
Agrobacterium	20	0	0	0	20	20	19	20	4

N: total number of strains.
Su: sulphonamide, Te: tetracycline, Ch: chloramphenicol,
Po: polymyxin, St: streptomycin, Ge: gentamycin,
Am: ampicillin, Ca: carbenicillin.

reaction, they belonged to Ørskov's biotypes 16 or 17. Among the strains of *E. cloacae* one was aberrant as regards the adonitol reaction; this was the strain isolated from monitor A.

Table 5 shows that few acquired antibiotic resistance characters were present in the more important potential human pathogens, i.e. *Enterobacteriaceae* and *P. aeruginosa*. The most striking characters were resistance to sulphonamide and ampicillin in strains of *K. pneumoniae* and *E. coli* respectively. Resistance to gentamycin was not observed. Multiple antibiotic resistance characters could be demonstrated in several strains of the other species.

DISCUSSION

The heat-treated monitors examined in this study constituted a heterogenous group, both as regards technical design and decontamination programme (E–H).

They were clearly superior to the chloramine-disinfected recirculating single-pass monitors (A–C), but scarcely less contaminated than the formalin-disinfected single-pass monitor D (Table 1). The lowest degree of contamination during dialysis was obtained with monitors G and H. They were characterized by the most intensive heat treatment programmes and were the only monitors with a temporary heating of the dialysate to 80°C during dialysis, both were of the single-pass type. In monitor E the heat treatment *per se* was efficient (Table 2), but during dialysis microorganisms proliferated rapidly and could reach high values towards the end of dialysis. This may be ascribed to the design of the monitor as a batch recirculating system; unlike the other monitor types no dialysate is exhausted during dialysis, and the concentration of waste products thus increases, reaching the highest values towards the end of dialysis. Once contaminated this monitor type constitutes an ideal incubator. The results obtained with monitor F point to an inefficient heat-disinfection procedure. The lack of increase in contamination during dialysis in this monitor and in monitor G may be associated with their design as single-pass monitors, but may equally well be due to a slowly growing microbial flora (Table 3). The finding of heavy contamination of the chemically-disinfected recirculating single-pass monitors (A–C) corresponds to the results of previous studies (7, 18). In these monitors the concentration of microorganisms was

TABLE 3 *Microorganisms Isolated from the Monitors at the End of the Dialyses*

Monitor type	A	B	C	D	E	F	G	H
Specimens contaminated	19	12	32	17	25	15	9	10
Number of isolates								
Escherichia coli	1	8	0	0	0	0	0	0
Klebsiella pneumoniae	14	0	0	0	1	0	0	3
Klebsiella oxytoca	0	0	0	0	1	0	0	0
Citrobacter freundii	11	0	0	1	0	0	0	0
Enterobacter cloacae	1	0	0	0	0	0	0	4
Pseudomonas aeruginosa	4	8	32	12	0	0	0	4
P putida (biotype 11)	0	0	2	0	15	3	0	0
Fluorescent *Pseudomonas* biotype 6	16	12	1	10	7	0	0	0
Other fluorescent Pseudomonads	0	0	3	0	2	0	0	0
P maltophilia	0	0	1	0	15	0	0	0
Other Pseudomonads	4	3	18	5	0	0	1	0
Alcaligenes	0	0	0	0	0	0	9	2
Aeromonas formicans	7	3	0	0	2	0	0	6
Xanthomonas	0	0	0	0	0	0	0	2
Acinetobacter calcoaceticus	0	0	0	0	0	0	0	5
Flavobacterium	0	0	0	0	2	0	0	0
Agrobacterium	0	0	0	0	4	16	0	0
Bacillus	0	0	0	0	1	0	0	0
Micrococcus	0	0	0	0	2	0	1	0

TABLE 4 *Biochemical Characteristics of Enterobacteriaceae Isolated from the Monitors at the End of the Dialyses*

	K pneumoniae 18 strains			*C freundii* 12 strains			*E coli* 9 strains			*E cloacae* 5 strains		
	+	(+)	−	+	(+)	−	+	(+)	−	+	(+)	−
Adonitol	18	0	0							1	0	4
Dulcitol	18	0	0	0	0	12	0	0	9	0	0	5
Inositol	18	0	0							0	0	5
Lactose				12	0	0	0	9	0	4	1	0
Raffinose				12	0	0	0	0	9			
Rhamnose							9	0	0			
Sorbose	0	0	18									
Sucrose				12	0	0	0	0	9			
Malonate				0	0	12				1	4	0
Urease	18	0	0	8	4	0				5	0	0
Lysine							0	0	9			
Arginine				12	0	0	0	9	0			
Ornithine				0	0	12	9	0	0			
D-tartrate	0	0	18							0	0	5
Citrate	12	5	1							5	0	0
Mucate	18	0	0							5	0	0
Hydrogen sulphide				12	0	0						

\+ positive within 2 days
(+) positive within 3 to 7 days
— negative after 7 days

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high from the start of dialysis, irrespective of whether the water supply was sterile dialysis fluid (monitor A and B) or deionized tap water (monitor C). The results obtained with monitor A (Fig. 2) indicate that these monitors were contaminated to a marginal degree at the end of dialysis, reaching a value of about 10^5 microorganisms per 0.1 ml dialysate, which is not far from the marginal value reached in stagnant dialysate (11). The degree of contamination and the repeated isolation of the same species (Table 3) and type (Table 4) from each monitor indicate that bacterial foci survive within the monitors between dialyses. The same may be true of the other monitor types, in particular monitor F but recontamination may also have occurred from outside, i.e. from the tubes leading the dialysate to the monitor. In monitor H the reused dialyser tubes were stored in a weak disinfectant. Probably they have become contaminated during the course of the observation period, thus being the focus of recontamination (Fig. 1) (12). The bacterial flora points to monitor A as a main source of contamination (Table 3 and 4).

The antibiotic sensitivity tests show that aquired resistance characters were rare among the potential human pathogens (Table 5). Among the other species the multiple antibiotic resistance characters probably reflect the natural state, e.g. the widespread resistance to the aminoglycosides, which was not observed among the potential human pathogens. Transmission of multiple resistance characters, as proposed by *Favero et al.* (7) seems to be only a minor problem. The results of this study indicate that the contamination of dialysis monitors is determined by several factors, including the design of the monitors, the decontamination procedures, the duration of the dialyses, and the flora contaminating the monitors. Heat treatment of monitors do not eliminate microbial proliferation during dialysis, but the introduction of monitor types with appropriate heat decontamination programmes may lower the level of microbial contamination.

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RAPID IDENTIFICATION OF GROUP A, B, C AND G BETA HAEMOLYTIC STREPTOCOCCI BY A MODIFICATION OF THE CO-AGGLUTINATION TECHNIQUE. COMPARISON OF RESULTS OBTAINED BY CO-AGGLUTINATION, FLUORESCENT ANTIBODY TEST, COUNTERIMMUNOELECTROPHORESIS AND PRECIPITIN TECHNIQUE

HEIKKI ARVILOMMI, OUTI UURASMAA and ANNIKKI NURKKALA

Arvilommi, H., Uurasmaa, O. & Nurkkala, A. Rapid identification of group A, B, C and G beta-haemolytic streptococci by a modification of the co-agglutination technique. Comparison of results obtained by co-agglutination, fluorescent antibody test, counterimmunoelectrophoresis, and precipitation technique. Acta path. microbiol. scand. Sect. B 86: 107–111, 1978.

A rapid modification of the co-agglutination (COA) technique for grouping A, B, C and G beta-haemolytic streptococci was developed. The results are obtained within three hours after inoculation from primary plates of 0.5 ml broth. This method was compared to the fluorescent antibody test (FA) and counterimmunoelectrophoresis (CIE), two other rapid methods available for serological grouping of streptococci. Of 71 recently isolated streptococcal strains from clinical sources, 70 were correctly grouped in COA, 43 in FA and 68 in CIE.
With commercial reagents COA compared favourably in accuracy to the other methods and can be recommended for routine serological grouping of beta-haemolytic streptococci in the clinical laboratory.

Key words: Beta-haemolytic streptococci; rapid identification; co-agglutination technique.

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Beta-haemolytic streptococci are among the most commonly occurring bacterial strains in clinical laboratories. Their identification does not usually pose problems. The presumptive grouping can be carried out with methods which are fairly well adapted to the routine work of the microbiological laboratory. One of these, and probably the most widely used, is Maxted's Bacitracin test (*Maxted* 1953), which differentiates group A from other streptococci on the basis of their different sensitivity to bacitracin. However, in a previous study on co-agglutination (COA) by one of us (*Arvilommi* 1976), the bacitracin test did not give satisfactory results. This, together with the observation of an unexpectedly high frequency of group B streptococci in the clinical material in our laboratory, prompted us to look into rapid methods of serological grouping of streptococci. We concentrated upon methods which 1) could be applied to material from primary blood agar plates, 2) could use commercial reagents, and 3) would provide results in a few hours. In this

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high from the start of dialysis, irrespective of whether the water supply was sterile dialysis fluid (monitor A and B) or deionized tap water (monitor C). The results obtained with monitor A (Fig. 2) indicate that these monitors were contaminated to a marginal degree at the end of dialysis, reaching a value of about 10^4 microorganisms per 0.1 ml dialysate, which is not far from the marginal value reached in stagnant dialysate (11). The degree of contamination and the repeated isolation of the same species (Table 3) and type (Table 4) from each monitor indicate that bacterial foci survive within the monitors between dialyses. The same may be true of the other monitor types, in particular monitor F, but recontamination may also have occurred from outside, *i.e.* from the tubes leading the dialysate to the monitor. In monitor H the reused dialyser tubes were stored in a weak disinfectant. Probably they have become contaminated during the course of the observation period, thus being the focus of recontamination (Fig. 1) (12). The bacterial flora points to monitor A as a main source of contamination (Table 3 and 4).

The antibiotic sensitivity tests show that aquired resistance characters were rare among the potential human pathogens (Table 5). Among the other species the multiple antibiotic resistance characters probably reflect the natural state, *e.g.* the widespread resistance to the aminoglycosides, which was not observed among the potential human pathogens. Transmission of multiple resistance characters, as proposed by *Favero et al.* (7) seems to be only a minor problem. The results of this study indicate that the contamination of dialysis monitors is determined by several factors, including the design of the monitors, the decontamination procedures, the duration of the dialyses, and the flora contaminating the monitors. Heat treatment of monitors do not eliminate microbial proliferation during dialysis, but the introduction of monitor types with appropriate heat decontamination programmes may lower the level of microbial contamination.

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containing beta-haemolytic streptococci from various clinical sources were analysed, 105 (60%) contained enough colonies for inoculating the broth. Of them, 5 (5%) failed to produce sufficient growth in 3 hours for the co-agglutination test. We also found that the streptococcal cultures can be heated at 100° C for 10 min without affecting the co-agglutination result. It is thus possible to work with killed suspension of streptococci if this is preferred. If the inoculum in the broth is not pure, some rapidly growing organisms might cause interference in the co-agglutination test. Therefore we tested some enterobacteria, staphylococci and also strains of alfa-haemolytic streptococci in the 3 hour co-agglutination test. Unexpected cross reactions may occur and lead to occasional false positive results as one Klebsiella strain reacted with G reagent. By careful subculturing and checking with gram staining this can be prevented.

TABLE 2. *Strains Giving Discrepant Results with One or More Techniques*

Strain No	Precipitation	CA	FA	CIE
462	A	A	*A C*	A
714	A	A	*A C*	A
2461	A	A	A	*A C*
379	B	B	*C*	B
624	B	B	*C*	B
755	B	B	*C*	B
783	B	B	*G*	B
325	G	G	C G	G
336	G	*B G*	G	*B G*
1014	G	G	G	*B*
1019	G	G	C G	G
No of inconclusive results		1	8	3

Comparative study

71 strains of beta-haemolytic streptococci from various clinical sources were collected and grouped by precipitation. These strains were then used in a comparative study in which they were independently grouped by three rapid methods COA, FA and CIE. The results are given in Table 1 When the three methods were compared to the precipitation test which in this study served as a standard method, it appeared that a discrepancy was found in 1/71 cases in COA, 8/71 in FA and 3/71 in CIE. The results which were not identical in all these tests are collected in Table 2 It is of interest to note that the group G strain which reacted with both B and G reagents in COA did so also in CIE.

We noticed difficulties in grouping because of cross reactions. This is a disadvantage, which seems to be a more or less common feature to all the methods described in this paper In the FA test, the group B antiserum had to be excluded because of cross reactions. We were unable to find a dilution which could differentiate group B streptococci from the others. Of group A antisera labelled with fluorescein, one (BBL) gave clearcut reactions and there were practically no cross reactions, while with the other there were many and the results obtained had to be discarded. Tables 1 and 2 show that most of the divergent results obtained with FA are apparently due to the cross reactions of group C antiserum. The antisera used in CIE gave plenty of cross reactions as well. This can probably be explained by the fact that the sera were prepared for capillary precipitation which does not detect as small amounts of cross reacting antibodies as the more sensitive CIE does.

TABLE 1 *Comparison of Co-agglutination, Fluorescent Antibody Test and Counter-immunoelectrophoresis to Precipitation*

Precipitation		COA					FA					CIE				
		A	B	C	G	NG[a]	A	B	C	G	NG	A	B	C	G	NG
A	20	20					18				2	19				1
B	29		29						3	1	25[b]		29			
C	4			4					4					4		
G	18				17	1				16	2		1		16	1
Total	71	20	29	4	17	1	18		7	17	29	19	30	4	16	2

[a] NG = non groupable

[b] Group B specific FA reagent had to be excluded. It did not differentiate group B streptococci from other

study the co-agglutination technique, described by *Christensen et al.* (1973), was modified in order to obtain grouping results within a few hours. It is compared to the fluorescent antibody test (FA) and the counter-immunoelectrophoresis technique (CIE).

MATERIALS AND METHODS

Streptococcal strains were collected from the cultures of specimens sent to the Public Health Laboratory. The samples were cultured on sheep blood agar plates (Blood Agar Base, Oxoid Ltd London, England substituted with 7% defibrinated sheep blood) and incubated aerobically overnight. Colonies of beta haemolytic streptococci were subcultured on blood agar plates. These cultures were used in the comparative study described below.

Precipitation

To determine the Lancefield group the group specific carbohydrate was extracted by autoclaving (*Rantz & Randall* 1955) and tested by double immunodiffusion as described earlier (*Arvilommi* 1976).

Co-agglutination

The method (*Christensen et al.* 1973) was modified as described in the Results section and Fig. 1. Commercial reagents, Phadebact® Streptococcus Test (Pharmacia Diagnostics AB Uppsala, Sweden) with anti-A,B C and G group specific activity were used. Trypsin was obtained from Difco (Trypsin 1:250 Difco Laboratories, Detroit, Michigan, USA).

Fluorescent Antibody Test

The method was essentially the one described earlier (*Arvilommi* 1976) except that instead of broth cultures, suspensions of beta-haemolytic colonies were made in a drop of saline on objective glass slides. Commercial fluorescein conjugated groups A, B C and G antisera were obtained from Difco and group A antiserum from BBL (Maryland USA).

Counter-immunoelectrophoresis

Streptococcal strains were inoculated into 2 ml of Todd Hewitt broth and incubated at 37° C water bath for 3 h. Carbohydrate extraction was performed by autoclaving as described earlier (*Arvilommi* 1976) and the extracts tested by CIE. In preliminary tests we could not obtain consistent grouping results by using cultures without polysaccharide extraction. A method described by *Pesendorfer & Krassnitzky* (1970) was followed. Briefly 1% agarose (Indubiose A37 L'Industrie biologique française S.A. Gennevilliers, France) in barbiturate buffer (0.13M pH 8.1) was used to prepare gel slabs of 1.7 × 100 × 100 mm size. Two rows of wells (Ø 3 mm) were punched 6 mm apart. The electrophoresis was run for 50 min at 60 V and 20–30 mA in a continuous buffer system with fourfold filter paper strips as bridges between the gel and the buffer vessels. The plates were inspected for precipitates immediately after the electrophoresis was stopped, and photographed for further reference. The antisera used were as follows. A, C and G from Difco and B from Behringwerke (Marburg, Germany).

RESULTS

Technical aspects

We tested several possible ways of obtaining a more rapid grouping result based on the existing co-agglutination method. Firstly direct co-agglutination of the colonies on the primary plates, according to *Edwards & Larson* (1974), did not work very well in our hands. Secondly colonies from the primary plates were suspended in a phosphate buffer on microscope slides and tested for co-agglutination. This technique gave inconsistent results as well. The outcome was not improved by trypsin treatment of the streptococcal suspension. Thirdly we studied the possibilities of shortening the incubation time after inoculation of Todd Hewitt broth. For this purpose the volume of the broth was reduced to 0.5 ml. Of 71 pure cultures, all grew in 2½ hours to provide sufficient antigen for the co-agglutination test.

The method thus developed is depicted in Fig. 1. Co-agglutination was carried out by placing a small drop of each reagent on a glass slide followed by a drop of broth culture. After rocking the slides for one minute the possible co-agglutination was recorded. The method was also tried in everyday practice. When 175 consecutive primary plates

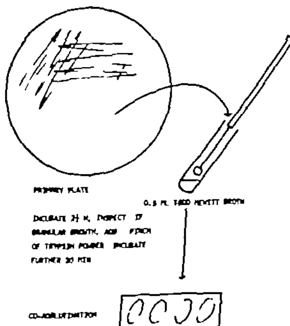


Fig. 1. Schematic representation of the modification of the co-agglutination technique.

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DISCUSSION

We have described a convenient modification of a method for serological grouping of streptococci in a clinical laboratory That 24-hour incubation of broth cultures for a co-agglutination test is not necessary in the majority of cases has been recently reported by *Farrel & Amirak* (1976) and *Rosner* (1977) who used 4-hour incubation. Generally 3–10 colonies are needed for inoculation of the broth for a succesful 3-hour test. The proportion of primary plates containing enough colonies varies depending on whether selective media are used, or whether plates containing only a few colonies are regarded as clinically significant. The proportion of 60% in the present study reflects the fact that we regarded even single colonies as significant and did not use selective media.

The methods of grouping streptococci are based on the pioneering work of *Lancefield* (1933) and have been extensively evaluated by *Facklam* in a recent review (1976). Classical capillary precipitin test and other precipitation techniques require the carbohydrate antigen to be extracted from the streptococci There are several methods for carbohydrate extraction However methods where this procedure can be omitted altogether are conceivably more appealing to the clinical microbiologist. Such methods are FA COA and, to a certain extent, CIE, the latter having been shown to work with group B streptococci without extraction (*Hill et al* 1975). Our experience is that CIE does not work well without extraction. This also accords with other researchers (*Wadström et al* 1974). Therefore we share the view of *Facklam*, who could not see the advantage of CIE over capillary precipitin with extracts.

TABLE 3 *Summary of the Properties of Co-agglutination, Fluorescent Antibody Test and Counter-immunoelectrophoresis in Quick Grouping of Beta haemolytic Streptococci*

	Accuracy	Extraction	Equipment	Time	Work
Co-agglutination	good	no	–	3 h	+
Fluorescent Antibody	variable	no	+ +	10 min–2 h	+
Counter immuno-electrophoresis	good?	yes	+	4–5 h	+ +

Immunofluorescence is used as a standard method to identify group A streptococci directly from throat swabs in many laboratories. It is a rapid method and fairly easy to perform, provided good antiserum is available. As noted in this study the quality of commercial antisera varies greatly The problems with cross reactions have also been reported by other investigators (*Watson et al.* 1975). Thus, commercial antisera of good quality seem to be rare. One apparent explanation may be the existence of true cross reactions, i.e. shared antigenic structure, which therefore make the elimination of cross reacting antibodies very difficult. Another explanation is the capacity of group A C and G streptococci to react nonspecifically with rabbit IgG (*Kronvall* 1973 *Christensen & Kronvall* 1974) by binding to the Fc part of this immunoglobulin. Quite recently this disadvantage was overcome by *Cars et al.* (1975) who used F(ab)$_2$ fragments in their FA tests with good results. These reagents, however are not commercially available. Some of the characteristics of the three methods are collected in Table 3 If the aim is to have a rapid and reliable method of grouping streptococci, CIE would be excluded because of the need for antigen extraction. COA and FA are therefore left for comparison. COA seems to be at least as accurate as FA. An FA test can be carried out rapidly but COA too, gives results in a few hours. The manipulation needed with both tests is insignificant. Assessment of co-agglutination on microscope slides is, however regarded as easier by many people than grading the intensity of fluorescence. No special equipment is needed for COA. The costs of these methods depend on the number of tests run each day and are difficult to estimate, but they can be lowered considerably by the economical use of the reagents.

We wish to thank *Pharmacia Diagnostics AB* for the supply of reagents.

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THE EFFECT OF COLOSTRUM AND COLOSTRAL ANTIBODY SIgA ON THE PHYSICO-CHEMICAL PROPERTIES AND PHAGOCYTOSIS OF *ESCHERICHIA COLI* O86

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Magnusson, K.-E., Stendahl, O., Stjernström, I. & Edebo, L. The effect of colostrum and colostral antibody SIgA on the physico-chemical properties and phagocytosis of *Escherichia coli* O86. Acta path. microbiol. scand. Sect. B, 86: 113–120, 1978.

A hydrophilic effect of human colostrum and colostral antibody SIgA binding on *Escherichia coli* O86 has been demonstrated by hydrophobic interaction chromatography on Octyl-Sepharose and partition in a polymer two-phase system containing dextran, poly(ethyleneglycol) and poly(ethyleneglycol)-palmitate. Furthermore, antibody SIgA binding reduced the negative surface charge of the *E. coli* bacteria. The reaction between centrifuged but not further purified colostrum and bacteria yielded a complex which, compared to bacteria alone, showed decreased negative and increased positive surface charges, the latter being sensitive to pepsin. Binding of SIgA or colostrum to *E. coli* showed no definite effect on the attachment to and phagocytosis by polymorphonuclear cells *in vitro*. The effects observed are discussed in relation to the structure of SIgA.

Key words: Colostral antibody SIgA; Escherichia coli O86; physico-chemical properties, phagocytosis.

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The distribution of immunoglobulins in the body varies according to the immunoglobulin class. It has long been known that human colostrum is especially rich in secretory IgA (SIgA, 7, 8, 21). As well as in the mammary glands, SIgA is synthesized primarily in relation to different mucous membranes and external secretions at sites which are connected directly with the outside world, and thus liable to contamination. In these sites it is relatively more abundant than IgG, whereas in the blood and internal secretions IgG dominates (21).

Binding of antibody IgG to microorganisms and other particles usually leads to complement activation and opsonization apparently aimed at destruction of the antigen. No such function is known for SIgA. As antiserum (23) and antibody IgG (5, 17–20) react with hydrophilic microorganisms, their surfaces become more hydrophobic, whereas the binding of SIgA seems to have an opposite effect (6). However, with methods then available, the physico-chemical nature of the effect of SIgA could not be analyzed accurately. The present investigation employs two methods, viz. partitioning in an aqueous two-phase system and hydrophobic interaction chromatography (9) for direct demonstration of tendency to charge and hydrophobic interaction. To obtain specificity in the reaction between antibody SIgA and bacteria, the cross-reaction between human blood group antigen B and *Escherichia coli* O86 was employed, and colostrum from non-B mothers was collected as a source of antibody SIgA (6).

MATERIALS AND METHODS

Preparation of Colostral SIgA

Colostrum was collected within 1–2 days *post partum*. It was centrifuged 2–3 times at 2500 G for 20 min. The fat at the top of the tube was removed and the sediment

ml distilled water for 20 s after which 2 ml 3.4% (w/v) PBS was added. The cells were washed twice in Krebs-Ringer phosphate buffer without Ca^{2+}. The percentage of PMN cells was not below 70% when used in the experiments. About 2×10^7 cells in 3 ml of the complete Krebs-Ringer phosphate buffer with Mg^{2+} and Ca^{2+} and 10 mM glucose (KRG) were added to tissue-culture petri-dishes (50 × 13 mm, Flow Laboratory) with five Millipore filters (HAWPO 1300, Millipore). The cells were allowed to settle for at least 30 min. Non-adhering material was removed by decanting and rinsing twice with KRG. 4 ml of KRG, with or without 10 µg/ml Cytochalasin B (Cyt B, EGA-Chemie KG 7924 Stenheim bei Heidenheim, Brenz, West Germany) was added and the dishes were incubated at 37° C for 20 min. Finally 5 ml of the suspension with ^3H leucine-labelled bacteria (2×10^9/ml) in PBS was added. After mixing, a sample (0.5 ml) was withdrawn and the zero-min filter taken. Filters were subsequently removed at 30, 60 (two) and 120 min, washed gently in three beakers with saline, dried, and finally put into a scintillation vial with 5 ml Aquasol (NEN) and 2 ml water. *Controls without PMN cells were made to study* the adhesion of the bacteria to the filters. Two parallel experiments were carried out.

Immunofluorescence

Bacteria sensitized with defatted colostrum were treated for 30 min at room temperature with FITC-conjugated anti-human IgA (Wellcome), IgG (SBL, Sweden), IgM (Wellcome) and C3 (Wellcome). They were washed once in PBS before being put onto microscope slides and examined in incident light with Zeiss Standard RA microscope. Two KP 500 filters and barrier filter 455 were used for excitation and condenser 510 and barrier filter 528 for emission.

RESULTS

Partition in Two-phase Systems

In the basal two-phase system *E. coli* O86 accumulated mainly at the interface and in the dextran-rich bottom phase (Table 1–3) and to a small extent in the PEG-rich top phase. Since *E. coli* O86 was transposed into the top phase by TMA-PEG but not by S-PEG (Table 1–3), negative charges seem to dominate *the electrostatic proper*ties of the bacterial surface. The bacteria were transposed to the top phase also by *P-PEG this* indicating accessibility of hydrophobic groups at the bacterial surface. However, in the latter case there was a discrepancy between experiments as regards the extent of the affinity. This was either considered as being due to changed growth conditions or solubility of P-PEG at partitioning. Reaction of *E. coli* O86 with colostrum reduced the partition to the bottom phase slightly in the basal two-phase system and also reduced the transposition by P-PEG and TMA-PEG thus indicating loss of or reduced accessibility of hydrophobic and negatively charged surface structures. The increased effect by S-PEG showed the acquisition of positively charged groups (Table 1).

After purification of SIgA from colostrum, IgG and IgM were not detected by immunodiffusion or by nephelometry. Exposure of *E. coli* to the SIgA preparation increased *the partition to the bottom* phase in the basal system (Table 2). The presence of TMA-PEG or P-PEG *in the phase system* had less

TABLE 1 *Aqueous Biphasic Partitioning* a) *of* E. coli *O86 Sensitized with Colostrum* b) *Expressed as percentage Bacteria* c) *in Top (T) and Bottom (B) Phase*

Fraction PEG-substitution of total PEG in phase system	O86		O86 + colostrum	
	T	B	T	B
None	3	44	1	18
TMA-PEG d)/(TMA-PEG + PEG) = 0.125	65	15	46	7
S-PEG e)/(S-PEG + PEG) = 0.125	3	34	57	1
P-PEG f)/(P-PEG + PEG) ≈ 0.04	26	26	15	16

a) One representative experiment (three experiments performed)
b) Immunoglobulin content not determined
c) Bacteria labelled with $Na_2{}^{51}CrO_4$
d) bis-trimethylamino-poly (ethyleneglycol)
e) bis-sulpho-poly (ethyleneglycol)
f) poly (ethyleneglycol)-palmitate

discarded. The interjacent liquid was frozen at −85° C until use. It was used as such either for sensitization of bacteria or for further preparation of SIgA. Except for the initial extra procedure to remove fat, the flow sheet for isolation of SIgA proposed by *Kobayashi* (12, see also 8) was followed. The purification was checked by immunodiffusion (Ouchterlony) with anti-human serum and antisera specific for the secretory component (SC) and the α- γ- and μ-chains (Behringwerke AG Marburg Lahn, West Germany or DAKO-Immunoglobulins A/S Copenhagen, Denmark). Only fractions were collected which gave precipitation lines with anti SC anti-α-chain, and anti-human whole serum and not with the other sera. The pooled fractions were dialysed against phosphate buffered saline, pH = 7.3 (PBS), and then stored at −85 C. The concentrations of IgA, IgG and IgM were determined either by the Mancinitechnique (15) or by nephelometry (13). With these techniques, the actual concentration of SIgA was probably underestimated by a three to four fold factor (7) since SIgA was not used for calibration.

Bacteria
The *E. coli* O86 was kindly supplied by Dr. *F. Orskov* (Statens Seruminstitut, Copenhagen, Denmark). The bacteria were grown overnight (16 h) at 37° C in 10 ml of the following enriched medium: glucose 1g, Na_2HPO_4 $2H_2O$ 0.6 g, NaCl 1.8 g, Bacto-Peptone (Difco) 10 g, beefextract (Difco) 5 g and deionized water 1000 ml. The pH was adjusted to 7.4. When metabolic labelling was used, 10 or 25 μl of (3,4,5)^{3}H leucine (NET 460 NEN West Germany) were pipetted into the 10 ml of the cultivation medium. Before use, the bacteria were washed four times in PBS. Labelling of bacteria with $Na_2{}^{51}CrO_4$ has been described elsewhere (16). The radioactivity was measured in a gamma scintillation counter (Intertechnique SA Plaisir France).

Sensitization of Bacteria with Colostrum and SIgA
The bacteria (1.5–2.0 × 10^9) were mixed with 1 ml defatted colostrum, incubated for 10 min, and then centrifuged at 2500 g for 20 min at room temperature. The pellet was resuspended and washed once in PBS. In the experiments with purified colostral SIgA, 1.5–2.0 × 10^9 bacteria were sensitized for 60 min at 37° C and then washed once in PBS. They were quantified by the absorbance at 650 nm as calibrated with viable count.

Two phase Partitioning
A two-phase system (1) was prepared from stock solutions of 20% (w/w) poly(ethyleneglycol) 6000 (= PEG- Carbowax 6000 Union Carbide, New York, N.Y.), 20% (w/w) dextran T 500 (Pharmacia Fine Chemicals AB Uppsala, Sweden), 0.1 M tris(hydroxymethyl) aminomethane buffer (= tris), pH = 7.0 and distilled water. The basal system contained 4.4% (w/w) PEG and 6.2% (w/w) dextran in 0.03 M Tris buffer. It was allowed to equilibrate at 4 C over night in a separation funnel. Then the bottom phase (rich in dextran) and the top phase (rich in PEG) were collected and stored separately at 4° C. To prepare phase systems for the partitioning studies, 2 ml of the bottom and 2 ml of the top phase were pipetted into graded test tubes. For tests with hydrophobic PEG 0.2 ml of PEG esterified with palmitic acid (= PEG-palmitate, P PEG) dissolved in PBS (0.13 mmol palmitic acid per g polymer 11) was added. The concentrations of P PEG are given in connection with the results. In the systems with charged PEG 12.5% of the PEG had been exchanged with either positively charged PEG bis-trimethylamino-($(CH_3)_3N^+$–) PEG (= TMA–PEG) or negatively charged PEG bis-sulpho (SO_3^-–) PEG (=S–PEG) (10) already in the preparation of the stock solutions.

0.1 ml of a suspension of bacteria was added to the graded tubes with the different phase systems, and the tubes were inverted (20 times) for mixing. They were then kept at 4 C for 45 min for separation of the phases. After reading the volumes of the bottom phase and the total system 0.5 ml aliquots were withdrawn from the two phases. After mixing with a Vortex homogenizer 0.5 ml was taken from the rest containing the material adhering to the interface. Quantification of the bacteria was made either by gammascintillation counter (Intertechnique SA Plaisir France) or by beta-scintillation counter (Isocap 300 Searle-Nuclear Chicago, Ill USA), as indicated in the captions to the tables. The distribution of bacteria was then calculated from volumes of the phases and concentration of bacteria in the samples taken.

Hydrophobic Interaction Chromatography
The interaction with hydrophobic column material (9) was investigated by adsorption to and elution from Octyl-Sepharose® (Pharmacia Fine Chemicals, Uppsala, Sweden). Characteristics such as bed volume, flow rate and composition of the eluant are given along with the results and in the captions accompanying the figures. A column K15/30 (Pharmacia Fine Chemicals) was used throughout the investigation. The elution pattern was read photometrically (at 650 nm) or by radioactivity which required in total about 10^{10} and 10^3–10^4 bacteria, respectively. In all experiments the bacteria were suspended in a 0.01 M phosphate buffer (pH = 6.8) with 1 M $(NH_4)_2SO_4$ and added to a column equilibrated with the same buffer. Elution was made by decreasing the concentration of $(NH_4)_2SO_4$ (from 1M to 0M) stepwise and concurrently increasing the concentration of Triton X–100 (from 0 to 0.1% v/v). No aggregation of the samples was observed before application onto the column.

In vitro *Assay of Phagocytosis*
Polymorphonuclear neutrophils (PMN cells) were obtained from guinea pigs after injection of 20 ml 0.1% (w/w) glycogen solution intraperitoneally (16, 17). The cells were collected 16 h later by rinsing the intraperitoneal cavity with saline containing heparin (15 I.U./ml, Vitrum, Stockholm, Sweden). The cells were then washed in Krebs Ringer phosphate buffer with 10 mM glucose but without Ca^{2+}, pH = 7.2. Contaminating erythrocytes were lysed by resuspending the pellet in 6

ml distilled water for 20 s after which 2 ml 3.4% (w/v) PBS was added. The cells were washed twice in Krebs-Ringer phosphate buffer without Ca^{2+}. The percentage of PMN cells was not below 70% when used in the experiments. About 2×10^7 cells in 3 ml of the complete Krebs Ringer phosphate buffer with Mg^{2+} and Ca^{2+} and 10 mM glucose (KRG) were added to tissue-culture petri-dishes (50 × 13 mm, Flow Laboratory) with five Millipore filters (HAWPO 1300, Millipore). The cells were allowed to settle for at least 30 min. Non-adhering material was removed by decanting and washing twice with KRG. 4 ml of KRG, with or without 10 µg/ml Cytochalasin B (Cyt B, EGA-Chemie KG 7924, Steinheim bei Heidenheim, Brenz, West Germany) was added and the dishes were incubated at 37° C for 20 min. Finally 5 ml of the suspension with ^3H-leucine-labelled bacteria (2×10^9/ml) in PBS was added. After mixing, a sample (0.5 ml) was withdrawn and the zero-min filter taken. Filters were subsequently removed at 30, 60 (two) and 120 min, washed gently in three beakers with saline, dried, and finally put into a scintillation vial with 5 ml Aquasol (NEN) and 2 ml water. Controls without PMN cells were made to study the adherence of the bacteria to the filters. Two parallel experiments were carried out.

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Fraction PEG-substitution of total PEG in phase system	O86		O86 + colostrum	
	T	B	T	B
None	3	44	1	18
TMA-PEG[d]/(TMA-PEG + PEG) = 0.125	65	15	46	7
S-PEG[e]/(S-PEG + PEG) = 0.125	3	34	57	1
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[a] One representative experiment (three experiments performed)
[b] Immunoglobulin content not determined
[c] Bacteria labelled with $Na_2{}^{51}CrO_4$
[d] bis-trimethylamino-poly (ethyleneglycol)
[e] bis-sulpho-poly (ethyleneglycol)
[f] poly (ethyleneglycol)-palmitate

TABLE 2. *Biphasic Partitioning* [a] *of E. coli O86 Sensitized with SIgA (40 μg/ml) Expressed as Percentage (± Range) Bacteria* [b] *in Top (T) and Bottom (B) Phase*

Fraction PEG-substitution of total PEG in phase system	O86 T	O86 B	O86 + SIgA T	O86 + SIgA B
None	4 ± 1	29 ± 1	5 ± 4	52 ± 2
TMA PEG/(TMA PEG + PEG) = 0 125	73 ± 1	4 ± 0	7 ± 1	29 ± 5
S-PEG/(S-PEG + PEG) = 0 125	1 ± 0	28 ± 3	1 ± 0	35 ± 2
P PEG/(P PEG + PEG) ≈ 0 04	8 ± 1	24 ± 0	1 ± 1	45 ± 1

a) Calculated from two experiments (two more experiments performed with another colostrum sample)
b) Bacteria labelled with (3 4,5)^{3}H leucine

effect on bacteria which had reacted with SIgA than on untreated bacteria, thus indicating a less negatively charged and less hydrophobic surface after coating with SIgA. S-PEG had no significant influence, thus possibly reflecting that the positive surface charge obtained with colostrum was due to binding of material other than SIgA. In order to ascertain whether the colostrum-derived material could be removed by enzyme treatment, colostrum sensitized *E coli* O86 were washed and incubated at pH 1.5–4 for 2 h at 37° C with pepsin (Sigma, 5 mg/ml in 0 1 M sodium acetate buffer Table 3). As in Table 1 colostrum treatment of the bacteria increased their partition to the interface in the basal two-phase system, increased the transposition to the top phase by S-PEG and almost annihilated the transposing effect of P PEG (Table 3) The affinity for S-PEG was lost after pepsin treatment pH 4 whereas in the basal system and in the system with P PEG the partition was almost unchanged. Pepsin treatment at pH 1 5 caused the partition to return close to the level of non-sensitized bacteria.

TABLE 3 *Effect of Pepsin at Different pH Values* [a] *on Aqueous Biphasic Partitioning* [b] *of E. coli O86 Sensitized with Colostrum* [c] *Expressed as Percentage Bacteria* [d] *in Top (T) and Bottom (B) Phase*

Fraction PEG-substitution of total PEG in phase system	Phase	O86 native bact	O86 pepsin pH 1 5	O86 + colostrum no treatment	O86 + colostrum pepsin pH 4	O86 + colostrum pepsin pH 3	O86 + colostrum pepsin pH 2	O86 + colostrum pepsin pH 1.5
None	T	2	1	2	1	2	1	2
	B	77	78	18	20	23	28	70
TMA PEG/(TMA PEG + PEG) = 0 125	T	56	57	58	71	73	80	86
	B	28	18	4	4	4	4	13
S-PEG/(S-PEG + PEG) = 0 125	T	2	1	65	10	3	2	2
	B	73	78	11	10	20	24	69
P PEG/(P PEG + PEG) = 0 04	T	12	11	11	10	16	18	15
	B	44	46	37	23	22	25	33

a) 37 C 2 h
b) One representative experiment (Three experiments performed)
c) Immunoglobulin content not determined
d) Bacteria labelled with $Na_2{}^{51}CrO_4$ overnight at 22° C

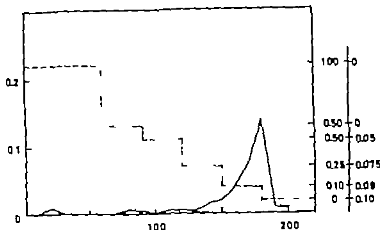


Fig. 1 Hydrophobic interaction chromatography of *E. coli* O86[(a)] sensitized with colostrum[(b)] on Octyl-Sepharose®

Ordinate (left) Absorbance, 650 nm; (right) Concentrations of $(NH_4)_2SO_4$ (M, left) and Triton X−100 (% v/v, right) in each elution buffer (0.01 M phosphate, pH = 6.8)

Abscissa Elution volume (ml)

[(a)]No. of bacteria in sample 1.5 × 10^{10} in 1.5 ml 1 M $(NH_4)_2SO_4$. Bed volume 28.3 ml. Flow rate 60 ml/h. Temperature 22° C. (—) O86, (   ) O86 + colostrum, (- - -) elution gradient. [(b)]Immunoglobulin content not determined

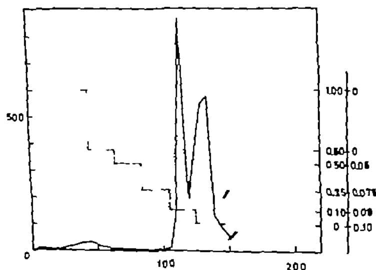


Fig. 2 Hydrophobic interaction chromatography of *E. coli* O86 [(a)]sensitized with SIgA (280 µg/ml) on Octyl-Sepharose®

Ordinate (left) Counts per min (cpm); (right) Concentrations of $(NH_4)_2SO_4$ (M, left) and Triton X−100 (% v/v, right) in each elution buffer (0.01 M phosphate, pH = 6.8)

Abscissa Elution volume (ml)

[(a)]No. of bacteria in sample 5 × 10^{8} in 0.5 ml 1 M $(NH_4)_2SO_4$, (3,4,5) ^{3}H-leucine-labelled. Bed volume 27.5 ml; Flow rate 75 ml/h. Temperature 22° C. (—) O86 (   ) O86 + SIgA, (- - -) elution gradient

TABLE 2 *Biphasic Partitioning[a] of* E. coli *O86 Sensitized with SIgA (40 µg/ml) Expressed as Percentage (± Range) Bacteria[b] in Top (T) and Bottom (B) Phase*

Fraction PEG-substitution of total PEG in phase system	O86 T	O86 B	O86 + SIgA T	O86 + SIgA B
None	4 ± 1	29 ± 1	5 ± 4	52 ± 2
TMA PEG/(TMA PEG + PEG) = 0 125	73 ± 1	4 ± 0	7 ± 1	29 ± 5
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a) Calculated from two experiments (two more experiments performed with another colostrum sample)
b) Bacteria labelled with (3 4,5)^{3}H-leucine

effect on bacteria which had reacted with SIgA than on untreated bacteria, thus indicating a less negatively charged and less hydrophobic surface after coating with SIgA. S-PEG had no significant influence, thus possibly reflecting that the positive surface charge obtained with colostrum was due to binding of material other than SIgA. In order to ascertain whether the colostrum-derived material could be removed by enzyme treatment, colostrum sensitized *E coli* O86 were washed and incubated at pH 1.5–4 for 2 h at 37° C with pepsin (Sigma, 5 mg/ml in 0 1 M sodium acetate buffer Table 3). As in Table 1 colostrum treatment of the bacteria increased their partition to the interface in the basal two-phase system, increased the transposition to the top phase by S-PEG and almost annihilated the transposing effect of P PEG (Table 3). The affinity for S-PEG was lost after pepsin treatment pH 4 whereas in the basal system and in the system with P PEG the partition was almost unchanged Pepsin treatment at pH 1.5 caused the partition to return close to the level of non-sensitized bacteria.

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Fraction PEG-substitution of total PEG in phase system	Phase	O86 native bact	O86 pepsin pH 1 5	O86 + colostrum no treatment	O86 + colostrum pepsin pH 4	pepsin pH 3	pepsin pH 2	pepsin pH 1 5
None	T	2	1	2	1	2	1	2
	B	77	78	18	20	23	28	70
TMA PEG/(TMA PEG + PEG) = 0 125	T	56	57	58	71	73	80	86
	B	28	18	4	4	4	4	13
S-PEG/(S-PEG + PEG) = 0 125	T	2	1	65	10	3	2	2
	B	73	78	11	10	20	24	69
P PEG/(P PEG + PEG) = 0 04	T	12	11	11	10	16	18	15
	B	44	46	37	23	22	25	33

a) 37° C 2 h
b) One representative experiment (Three experiments performed)
c) Immunoglobulin content not determined
d) Bacteria labelled with $Na_2{}^{51}CrO_4$ overnight at 22° C

DISCUSSION

In previous experiments on the effect of colostral SIgA on the partition of *E. coli* O86 it was shown that antibody SIgA reduced the negative charge of the bacteria (6). In this report two newly developed methods, viz. aqueous two-phase systems with added hydrophobically substituted polymers (11) and hydrophobic interaction chromatography (9), have been employed for direct examination of the hydrophobic/hydrophilic properties of *E. coli* O86 sensitized with colostrum or SIgA.

A general drawback in using colostrum for sensitization or as a source of SIgA is the limited amount of colostrum obtainable from one donor. This greatly reduces the possibility of making repeated experiments on the same lot of material (see, for instance, Tables 1 and 3).

As demonstrated previously (6), sensitization with colostrum reduced the negative charge of *E. coli* and added positive charges, so that influence on the partition was reduced for TMA-PEG and increased for S-PEG. By contrast, colostral SIgA reduced the negative charge without acquisition of positive groups, thus indicating shielding of the bacterial charge and little own charge of bound SIgA itself. Since the positive charge effect of colostrum was lost after pepsin treatment at pH 2–4, a peptidecontaining substance in colostrum seems to have become involved with the bacteria and SIgA. After pepsin treatment at pH = 1.5 most of the effect of colostrum was abolished (Table 3). We have not investigated further whether this was due to digestion, denaturation or elution. Both in partition experiments with PEG-palmitate and in hydrophobic interaction chromatography on Octyl-Sepharose, *E. coli* showed a tendency to hydrophobic interaction. After treatment with colostrum or SIgA, this property was impaired to a large extent (Tables 1 & 2, Figs. 1 &2). This change might be a consequence of, for instance, (a) a rearrangement of surface entities of the bacteria or (b) the physicochemical properties of the bound SIgA molecule. Rearrangement within the outer bacterial membrane seems less likely to be responsible for the change in physico-chemical properties, since the temperature during the experiment was rather low about 22° C and the effect of antibody SIgA was opposite to the hydrophobic effect of antibody IgG (17–20). The functions of the Fab portions of SIgA and IgG are similar although SIgA is tetrameric and IgG dimeric with respect to Fab. However there are great differences between the other parts of SIgA and IgG. A model of the three dimensional structure of IgG has recently been proposed, largely on the basis of amino acid sequence analysis and X-ray crystallography (3). The structure of SIgA is less well known, but its composition of two IgA molecules joined by a J-chain at the Fc terminals and the secretory component (SC) coiled around the two opposed Fc fragments has become established (4,8,21). As moieties with the capacity to produce gross hydrophilic effects, carbohydrate structures are of primary interest. There is a marked difference in the carbohydrate concentration between IgG and SIgA, 2–3% and 10–12.5% respectively. Furthermore human IgA is more easily soluble in water than IgG, which is a direct measure of hydrophilicity (8, 24). There is also a difference with respect to localization of the carbohydrate in the Ig molecules. In IgG the oligosaccharide site is in segment b of the $C\gamma2$ domain. Thus, most of it seems to be localized between the two H chains of the molecule so that little is exposed to the surface of the molecule (3). In the IgA-oligosaccharide site of the $C_\alpha2$ domain, the carbohydrate is supposed to point outwards from the IgA molecule, and there are also additional sites in the hinge region and in the tail piece where they are also likely to be exposed (3). For SIgA, the model described previously suggests a great influence of the SC (4) on the surface of a particle sensitized with antibody SIgA. Since the carbohydrate concentration of the SC, 15.6% (12) and 11.6% (22), is higher than for other constituents of SIgA, we suggest that it is mainly responsible for the hydrophilic effect observed (Fig. 1 &2, Table 1 &2).

It has been proposed that the biological function of SIgA is to eliminate antigen from mucosal tissue by preventing uptake (2,8). However the effect of colostrum and antibody SIgA on *E. coli* O86 with respect to attachment and phagocytosis was not significant. Rather than a dysopsonizing effect, an opsonizing effect by *SIgA* was observed. Three main reasons for this observation may be considered: (a) *E. coli* O86 is too poorly phagocytosed in the absence of antibody to allow clear-cut demonstration of a possible dysopsonizing effect by SIgA. (b) Contamination of the SIgA preparation with small quantities of IgG or IgM might obscure the effect of SIgA (25); (c) *SIgA* does not affect phagocytosis but causes the bacteria to become mucophilic. Affinity of SIgA for different components of mucus such as the glycoprotein mucin, amylase and lactoferrin, is known from frustrations in separation work (8). These propositions must be challenged further experimentally.

The excellent technical assistance of Mrs. *Elinor Gramerdin* and Miss *Kerstin Hagersten* is gratefully acknowledged. This work was supported by grant B77-16X-02183-11A from the *Swedish Medical Research Council*.

TABLE 4 *Effect of Colostrum[a] on Phagocytosis (% of Added Activity per Filter[b]) of E. coli O86[c] by PMN Cells and Adhesion to Filters after Different Incubation Periods*

System	30 min	n[d]	60 min	n	120 min	n
086 + PMN	0 03 ± 0 01	2	0 08 ± 0 02	4	0.39 ± 0 04	2
086 + PMN + colostrum (1:2)	0 05 ± 0 01	2	0 15 ± 0 03	4	0.32 ± 0 07	2
086 + PMN + colostrum	0 10 ± 0 01	2	0 19 ± 0 05	4	0.38 ± 0 04	2
086	0 02	1	0 06	1	0 08	1
086 + colostrum	0 02	1	0 03	1	0 06	1

a) IgA = 9 0 g/1 IgG = 1 2 g/1 IgM = 1 0 g/1
b) Activity ± range (n = 2) or ± S D (n ≥ 3)
c) Bacteria metabolically labelled with (3 4,5)^{3}H leucine
d) n = number of samples

Hydrophobic Interaction Chromatography (HIC)
Passing a suspension of *E coli* O86 in 1 M ammonium sulphate through a column with Octyl Sepharose retarded the bacteria almost completely (Fig. 1–2) Only when the ammonium sulphate concentration was reduced to 0 1 M with a concomitant increase in the Triton X–100 concentration to 0 09% were the bacteria eluted from the column. After treatment with colostrum, the largest fraction of bacteria did not adhere to the column. Smaller fractions were eluted subsequently even with up to 0 1 M ammonium sulphate and 0 09% Triton X–100 thus indicating heterogeneity among the particles. Similar results indicating even more heterogeneity were achieved with the purified SIgA fraction (Fig 2).

Phagocytosis
The phagocytosis by PMN cells of *E coli* was not changed after treatment with colostrum (Table 4). Adhesion of *E coli* to the filters in the absence of PMN was small and was scarcely affected by colostrum. Neither was the effect of the SIgA fraction conspicuous on attachment and phagocytosis (Table 5) although SIgA appeared to increase the interaction after both 60 min and 120 min. However this observation could not be justified statistically The phagocytosis was slow in all experiments, probably reflecting a limited liability of *E coli* O86 to be phagocytosed by PMN cells. In fact, not until the bacteria had been incubated with the PMN for 120 min was the uptake apparent. The interaction during the second hour was partly blocked by cytochalasin B (Table 4) indicating blocking of phagocytic activity of the PMN cells (14).

Immunofluorescence
Bacteria which had been sensitized with defatted colostrum and then treated with FITC-conjugated antisera specific for IgA IgG IgM or C3 showed strong fluorescence only with the anti IgA serum. Very weak fluorescence was obtained with the other antisera.

TABLE 5 *Effect of SIgA on Phagocytosis (% of Added Activity per Filter[a]) of E. coli O86[b] by PMN Cells and Adhesion to Filters after Different Incubation periods*

System	30 min	n[c]	60 min	n	120 min	n
086 + PMN	0 14 ± 0 01	2	0 14 ± 0 05	4	0 32 ± 0 09	2
086 + PMN + 70 µg/ml SIgA	0 13 ± 0 02	2	0 18 ± 0 06	4	0 47 ± 0 14	2
086 + PMN + 140 µg/ml SIgA	0 11 ± 0 02	2	0 23 ± 0 06	4	0 52 ± 0 17	2
086 + PMN + 10 µg/ml Cyt B	0 12 ± 0 01	2	0 20 ± 0 11	4	0 19 ± 0 03	2
086	0 05	1	0 05 ± 0 01	2	0 10	1
086 + 140 µg/ml SIgA	0 03	1	0.03 ± 0 01	2	0 06	1

a) See[b] Table 4
b) Bacteria metabolically labelled with (3 4,5)^{3}H-leucine
c) n = number of samples

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MICROCALORIMETRIC MEASUREMENT OF NORMAL AND ADENOVIRUS INFECTED HeLa CELLS

KARIN LJUNGHOLM, INGEMAR WADSÖ and LARS KJELLÉN

Thermochemistry Laboratory, Chemical Center, University of Lund, Box 740, S-220 07 Lund, Sweden, and Department of Virology, Institute of Medical Microbiology, University of Lund, S-223 62 Lund, Sweden

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Key words: HeLa cells, adenovirus infected, microcalorimetric measurements; heat effect.

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A twin microcalorimeter of the heat conduction type was used. The instrument and its operation have been described in detail elsewhere (*Spink & Wadsö* 1976, *Wadsö* 1974). The samples were enclosed in stainless steel ampoules, volume 7.5 ml. In all cases the calorimeter was thermostated at 37°C. The voltage signal was amplified with a Keithley 150 B Microvolt Ammeter (10 μV range) and was recorded by means of a Servogor RE 511 recorder (100 mV range). The baseline stability for the instrument was 1.5 μW/24 h.

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Calorimetric Experiments

The calorimetric ampoules were sterilized by autoclaving at 121° C for 0.5 h. The test ampoule was charged with 3 ml of a cell suspension, leaving an air phase of 4.5 ml. The reference ampoule was charged with the same amount of sterile medium.

The ampoules were pre-thermostated for 5 min at 37° C before being presented to the calorimeter. They were kept for 10 min in the thermostating positions after which they were inserted into the measurement zone.

In preliminary series of experiments the calorimetric ampoules were charged with HeLa cells suspended in standard medium at a concentration of 1.5×10^8 cells/l. The working procedure was carefully standardized but the heat effect curves obtained were still unpredictable. The following modification of the procedure, employing stavation treatment, was therefore adopted.

A calorimetric ampoule containing 3 ml of HeLa cells at a concentration of 1.5×10^8 cells /l, was incubated statically for 16–18 h at 37° C. During the incubation 90–95% of the cells adhered to the bottom of the ampoule, area 2.5 cm^2. The cells were washed once with MEM and 3 ml of starvation medium was added before the ampoule was presented to the calorimeter. After about 24 h, when stabilized heat effect curves had been obtained, the starvation medium was exchanged for standard medium or virus suspension.

Virus suspended in TRIS buffer pH 7.4 was added to the calorimetric ampoule at about 2000 PFU/cell. The virus particles were allowed to adsorb under static conditions for 1 h at room temperature. The buffer was then replaced with standard medium and the calorimetric measurement was continued.

In addition of the calorimetric measurements parallel experiments were performed with ampoules kept at 37°C outside the calorimeter. Cells from such ampoules were harvested by trypsin treatment following each step of the calorimetric experiment. The cells were counted and tested for survival ty trypan blue exclusion.

pO_2 Measurements

In several experiments the oxygen content of the culture medium was estimated using a membrane covered Ag/Pb oxygen electrode (*Borowski & Johnson* 1967).

Calculation of Results

Heat effect values are expressed as mW/l or as pW/cell (pW = 10^{-12}W). Uncertainty values are standard deviations.

RESULTS

Preliminary experiments

The calorimetric curves, the »thermograms«, obtained in the preliminary experiments are summarized in Fig. 1. The poor reproducibility may be due to heterogeneity in the cell materials, in particular differences in growth phases. As a means to overcome this problem we adopted a technique

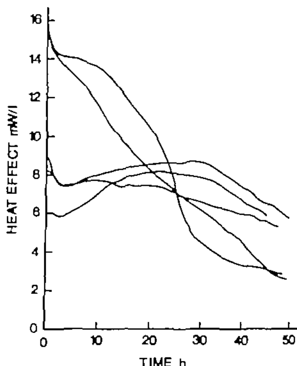


Fig. 1. Results of preliminary calorimetric experiments with HeLa cells grown in standard medium.

described by *Pardee* (1974), who employd starvation treatment of 3T3 cells in order to force them into the same growth phase.

HeLa cells in Starvation Medium

The thermograms obtained with starvation medium, and with all cells attached to the bottom of the ampoule are shown in Fig. 2. From about 24 h and onward the curves show the same pattern characterized by a linear decrease in the heat effects. The heat effect varied between the individual experiments, but the spread decreased by about one half

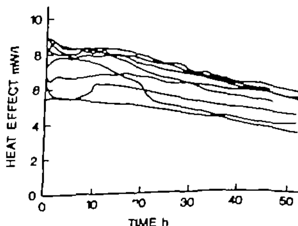


Fig. 2. *Thermograms for HeLa cells in starvation medium.*

TABLE 1 *Percentage of Dead Cells at 0, 24 and 48 h in Starvation Medium*

Time h	Dead cells %	Number of experiments
0	7 ± 2	19
24	10 ± 3	19
48	14 ± 4	3

The cells were treated with trypan blue at 0,24 and 48 h and examined under a microscope. The mean number of cells leaky to trypan blue was determined

when corrected for the number of cells in the samples.

In Table 1 results of viable counts conducted in parallel with the calorimetric experiments are summarized. After 48 h in the calorimeter 14 % of the cells were leaky to trypan blue. This decrease in viability is significantly less than the decrease in heat effect, about 30 % recorded over the same period. No cell proliferation was observed.

It is not known if the cells were in the same growth phase at the end of the 24 h starvation period. Preliminary results, (*Kjellén*, unpublished) demonstrate that HeLa cells differ from 3T3 cells used by *Pardee* in rehabilitation after starvation. Nevertheless the starvation treatment was a successful means by which reproducible thermograms could be obtained and by which a starting point for further calorimetric studies of HeLa cells was achieved.

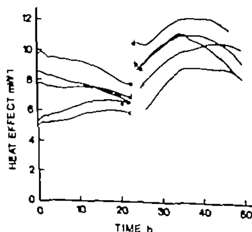


Fig. 3 Thermograms for HeLa cells in starvation medium (0–24 h) and standard medium (24–48 h)

Non-infected HeLa Cells

In one series of experiments HeLa cells were exposed to starvation medium for about 24 h and were then placed in standard medium. The thermograms are shown in Fig. 3. The heat effects observed immediately after the change to standard medium was poorly correlated with corresponding values at the end of the starvation period. The heat effects increased markedly after the change to the standard medium and a broad maximum was reached after 12–16 h.

The number of cells doubled during the 24 h exposure to the standard medium. About 5 % of the cells were leaky to trypan blue staining at the end of the experiment (Table 2).

TABLE 2 *Percentage of Dead Cells at 48 h. The Cells had been kept in Starvation Medium during 0–24 h*

Medium	Dead cells %	Number of experiments
Standard	5 ± 1	6
Standard + virus	12 ± 0	3

The cells had been kept in starvation medium during 0–24 h as described in Materials and Methods. At 48 h the cells were treated with trypan blue and the mean numbers of leaky cells were determined

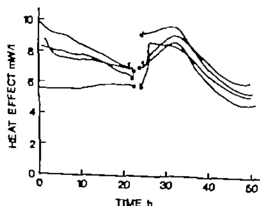


Fig. 4 Thermograms for HeLa cells in starvation medium (0–24 h) and standard medium containing adenovirus (24–48 h)

Virus Infected HeLa Cells

After starvation treatment for 24 h HeLa cells were infected with virus suspended in standard medium. The thermograms are presented in Fig. 4. As with non-infected HeLa cells the heat effect increased markedly after the change to complete

medium (see Fig 3) However a maximum was reached earlier about 8 h after the change of medium and the following decline was more rapid than in the experiments with non-infected cells. About 20 h after the maximum was reached the thermograms levelled out at values similar to those found at the end of the starvation period.

The infected cells did not show significant growth 12% of the cells were dead (Table 2) at the end of the test period

As with non-infected cells there was a poor correlation between heat effects observed immediately before and after the change of medium.

Tests for Oxygen in the Culture Medium

In a few experiments the oxygen potential in the culture medium was measured. After growth for 6 h in standard medium in a calorimetric ampoule the oxygen potential was close to 100% of the air saturation value. After 25 h the electrode measurements indicated that about 60% of the oxygen was consumed.

In some experiments with cells in standard medium the calorimetric observations were interrupted several times during the period 5–25 h and the liquid phase was aerated by inverting the ampoule several times. In other experiments the ampoules were opened and carbogen was briefly bubbled through the medium When the calorimetric measurements were resumed no significant change in the heat effects were observed. The results thus indicate that the calorimetric experiments were performed under aerobic conditions

DISCUSSION

We conclude that the heat production from about 5×10^5 HeLa cells attached to a solid support is within the sensitivity of the present microcalorimetric technique. The average heat effect value was found to be in the range of *20–40 pW/cell* depending upon the state of the cell

The results obtained in the preliminary series of experiments are significant as they show that the metabolic activity in HeLa cell preparations can vary to a very large extent even if the experimental conditions are carefully controlled.

Experiments with non-infected and infected cells, respectively showed significantly different thermograms In the former case a broad maximum was observed 12–16 h after the change of medium, whereas relatively sharp maxima were found already after about 8 h when virus was present. It is known that viral DNA replication is still at an early phase at that time (*Kjellén* 1962) and that the production of viral capsomers has hardly started. Maturation of virions takes place from 16 to 30 h after infection. We can thus conclude that there is a large impact on the cell metabolism far ahead of the maximum production of viral constituents.

It was shown that the present calorimetric experiments were performed under aerobic conditions. However in order to secure aerobic conditions during very long calorimetric experiments it may be advantageous to use a »perfusion« technique (*Wadsö* 1974 *Spink & Wadsö* 1976 *Kemp* 1975) where the cells can be continuously supplied with new medium

The method presented is believed to be useful for monitoring of the overall metabolic activity of tissue cells e.g in the detection of heterogeneity between cell samples, in studies of the effect of different media and as a timer for events in the cell cycle. However it is evident that much methodological work remains to be done on the correlation between the processes and the thermograms.

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COMPARISON OF SMALL HEPATITIS B SURFACE ANTIGEN PARTICLES AND HUMAN SERUM LOW DENSITY LIPOPROTEIN MOLECULES BY ELECTRON MICROSCOPY

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The small spherical particles associated with hepatitis B surface antigen (HB_sAg) could be distinguished from the low density lipoprotein (LDL) molecules in human serum by examination of coded, negatively stained preparations. The HB_sAg associated particles showed a more marked contrast against the background than LDL. Addition of specific antiserum to LDL caused a significantly reduced mean diameter of LDL molecules. An insignificant reduction in size of HB_sAg particles was found by corresponding treatment. It is suggested that the antibody molecules protect LDL molecules against artifical flattening during preparation.

Key words: Hepatitis B surface antigen; low density lipoprotein; electron microscopy; negative staining.

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Three morphologically distinct particles have been found to be associated with the hepatitis B surface antigen (HB_sAg) (3,5): Small 20 nm spherical particles, larger 42 nm spherical (Dane) particles and various tubular structures.

In previous studies (14, 15), we based identification of HB_sAg, after direct examination of diluted serum samples, on the presence of tubular and Dane particles. As the smaller HB_sAg associated particles closely resembled LDL of human serum (15), observation of free 20 nm spherical particles was not considered definite proof of the presence of HB_sAg. However, during these studies, a difference with respect to negative staining properties of LDL molecules and small HB_sAg associated particles was suspected.

The present paper describes experiments conducted to elucidate this possibility and to evaluate the cause of these different appearances.

MATERIALS AND METHODS

Human sera. Sera negative for HB_sAg (judged by double diffusion tests) were obtained from healthy individuals and used within a few days.

HB_sAg containing serum. Serum from an apparently healthy carrier of HB_sAg was used. This serum sample, which was known to contain few tubular and Dane particles, had been stored at −40°C.

Antisera. Specific antiserum against HB_sAg (anti-HB_s) was obtained from a patient given multiple transfusions. The serum sample had been stored at −40°C. Rabbit antiserum to human β-lipoprotein (LDL) obtained from Behringwerke A.G. (Op. /Batch No 1955 and K/Batch No. 2076) was also used. A human serum sample without any antibodies served as negative control. This serum sample had been stored under the same conditions as the anti-HB_s serum.

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Double diffusion experiments. These tests were carried

out as described previously (16). Tests for the presence of HB_sAg were performed in 1 per cent agarose gels (Agarose, L'Industrie Biologique Française S.A., Lot 7547), while the tests for the presence of LDL were carried out in 1 per cent Oxoid »Ionagar« No 2 (Oxoid Ltd., Batch No 239 1053).

Preparative ultracentrifugation. Preparative ultracentrifugation was carried out according to the method described for lipoproteins by *Havel et al.* (8) in a Beckman L50 preparative ultracentrifuge at 4°C. To obtain lipoprotein-free serum fractions lipoproteins floating at the density 1.21 g/cm³ were separated from the heavier serum proteins by ultracentrifugation at 105 000 g for 40 hours. Lipoproteins with densities between 1.050 and 1.080 g/cm³ were prepared by initial centrifugation at 176 000 g for 24 hours at a solute density of 1.050 g/cm³. The density of the infranatant solution was raised to 1.080 g/cm³ and centrifugation was performed at 176 000 g for 48 hours.

Preparation of pure »high-density« LDL fractions. Lipoproteins with densities between 1.050 and 1.080 g/cm³ were applied on a retrograde Bio-Gel® A 5m, 200–400 mesh (Bio-Rad Laboratories) column to obtain a pure homogeneous preparation of LDL molecules with a density as similar as possible to that of HB_sAg. The diameter and height of the column were 2.5 and 95 cm, respectively, and elution was performed using a 0.1 M Tris buffer at pH 8.2 containing 0.15 M NaCl and 0.001 M EDTA. The fractions shown by double diffusion tests to contain pure LDL were pooled, concentrated and dialysed.

Dialysis. The fractions obtained from these ultracentrifugation and column chromatography experiments were dialysed against several changes of 0.85 per cent NaCl at 4°C prior to electron microscopy.

Preparation of samples for electron microscopy. Serum samples and lipoprotein-free serum fractions from healthy individuals and from the HB_sAg carrier were diluted 1:4 in 0.85 per cent NaCl, while lipoprotein-free antiserum fractions (including the control serum) were diluted 1:9 prior to electron microscopy. Lipoprotein-free antiserum was added to both the sera and the fractions to be examined. The ratio of the test sample to the antiserum was varied until optimal aggregation was achieved. The mixtures were incubated at 37°C, tilted every tenth minute for one hour, and finally incubated overnight at 4°C. Samples containing lipoproteins were stored at 4°C and examined in the electron microscope within a few days. Other samples were kept at −20°C until examination.

Coding of samples was performed by a person who was not participating in the study, and the code was broken only after the samples had been finally scored.

Electron microscopy. The samples were negatively stained by use of a 3 per cent sodium phosphotungstate solution at pH 7.4, as described previously (14). A Siemens 1A electron microscope (accelerating voltage 80 kV, objective aperture 50 µm) or a JEM 100C (Jeol) electron microscope (used as transmission electron microscope, accelerating voltage 80 kV, objective aperture 60 µm) was used at instrumental magnifications of 120 000 × and 130 000 ×, respectively.

As the number of tubular and Dane particles was very small in the serum containing HB_sAg, decisions as to whether or not HB_sAg was present could be made before any such structures were observed.

Measurement of particle diameter and statistical analysis. Photographically enlarged (× 3) electron micrographs were used. The particle diameters were determined to the nearest tenth of a millimeter by use of a caliper.

As the distribution of particle diameters appeared to be normal (see below), Student's t test was used to compare differences between mean diameters.

EXPERIMENTS AND RESULTS

Attempts to Distinguish Small HB_sAg Associated Particles from LDL Molecules in the Electron Microscope

During previous studies (14, 15), a possible difference with respect to negative staining properties of LDL molecules and small HB_sAg associated



Fig. 1. Electron micrograph showing a higher degree of contrast surrounding a HB_sAg associated particle (A) than that of a lipoprotein molecule (B). Negative staining. Magnification × 360 000.

particles was noticed. HB_sAg associated particles appeared to contrast more strongly with the background than LDL molecules (Fig. 1). An experiment was therefore performed to ascertain whether this observation could be used to distinguish the two types of particles.

Three aliquots of diluted whole serum and three aliquots of the diluted lipoprotein-free fraction from each of five individuals were given to a person who coded 20 of the 30 samples. At least one aliquot of each sample from each person was coded, otherwise the author did not know the number of samples from any individual included among the 20 specimens. The antiserum fractions were coded separately. The antisera were added to the 20 test samples in separate experiments. Altogether 80 different samples were examined by electron microscopy.

The content of the samples, as determined by electron microscopy prior to the addition of antisera, is shown in Table 1. HB_sAg was observed in all samples obtained from the healthy carrier of this antigen. In addition, structures resembling this antigen were also found in one sample obtained from another individual. This was the only whole serum aliquot included from this person. A few free structures thought to be HB_sAg associated particles were, however, also seen in this sample after addition of anti-LDL serum or control serum. HB_sAg was not observed in the two lipoprotein-free serum fractions from this individual. Lipoprotein particles were recorded in all samples (Table 1), also in the "lipoprotein-free" fractions, although the content of lipoprotein molecules in the latter specimens was small.

The aggregation of LDL molecules was observed in all samples after addition of anti LDL serum. Likewise, HB_sAg associated particles were aggregated after addition of anti-HB_s serum in all samples from the HB_sAg carrier. Anti-HB_s serum also aggregated particles in the serum aliquot obtained from the individual mentioned above. Neither LDL molecules nor HB_sAg associated particles were aggregated in samples where control serum was substituted for antiserum.

Size Determination of «High Density» LDL Molecules and HB_sAg Associated Particles without and with Antibody Attached

The LDL obtained after column chromatography was submitted to electron microscopy. The diameter of 200 LDL particles from each of four samples was determined before and after the addition of specific antiserum.

The diameters of both free and aggregated LDL molecules appeared to be approximately normally distributed (Fig. 2). Corresponding distribution patterns were obtained for the four individual samples. The mean diameter of free LDL molecules was 21.4 nm ± 2.2 nm (mean ± S.D.), while aggregated molecules had a mean diameter of 19.9 nm ± 2.0 nm. The reduction in particle size observed after addition of specific antiserum (7.0 per cent) was significant ($t = 14.618$, $p < 0.0005$). Similar significant reductions in molecule diameters were observed in all individual LDL samples.

Small HB_sAg associated particles were examined in the same way. Altogether 83 free, presumably HB_sAg associated particles were measured; the distribution of the particles according to their diameters is shown in Fig. 3. A mean diameter of 21.2 nm ± 1.9 nm was found. The mean diameter

TABLE 1. *Scoring by Electron Microscopy Following Negative Staining of Coded Whole Serum and Lipoprotein Free Serum Samples (Density > 1.1 g/cm³) with Respect to Presence of Small HB_sAg Associated Particles and LDL Molecules, Respectively*

Samples scored by electron microscopy as containing	Number of samples from			
	normal individuals		HB_sAg carrier	
	Whole serum	Lipoprotein-free serum	Whole serum	Lipoprotein-free serum
HB_sAg	1 (0)[a]	0 (0)	1 (1)	2 (2)
LDL	9 (9)	8[b] (0)	1 (1)	2[b] (0)
Number of samples examined	9	8	1	2

[a] Expected values are shown in brackets.
[b] Only a small amount of LDL was present in these samples.

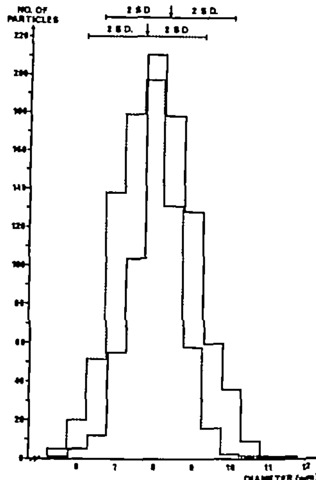


Fig 2 Distributions according to diameter of native LDL molecules (full line) and LDL molecules aggregated after addition of specific antiserum (dotted line) The measurements were made on electron micrographs (magnification × 390 000). Mean diameters (arrows) ± 2 S. D (21 4 nm ± 4 4 nm and 19 9 nm ± 4 0 nm) are indicated.

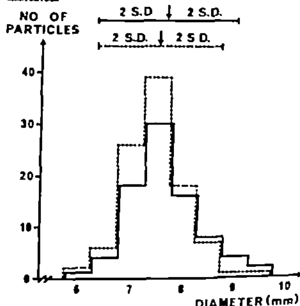


Fig 3 Distributions of free (full line) and antibody aggregated (dotted line) small spherical HB_sAg associated particles by diameter (magnification × 360 000). Mean diameters (arrows) ± 2 S D (21 2 nm ± 3 8 nm and 20 8 nm ± 3 2 nm) are indicated

of 100 aggregated HB_sAg associated particles was 20 8 nm ± 1 6 nm (Fig. 3). The reduction in diameter observed after addition of antiserum (1.9 per cent) was not significant (t = 1 785 d.f. 181 0 05 < p < 0 10).

DISCUSSION

Electron microscopy reveals a close resemblance between small HB_sAg associated particles and human serum LDL molecules. Both structures are spherical and have an appearance suggesting a substructure (2 3 6 11). The size of the particles is also similar Diameters of between 20 and 22 nm have been found for the HB_sAg associated particles (2 3 4 10 12 and others), while diameters of human serum LDL reported in the literature range from 19 3 to 21 6 nm (6 11 17) The dimensions of free HB_sAg associated particles and of free LDL molecules reported here (21 2 nm and 21 4 nm, respectively) agree well with these values.

In spite of these resemblances, it was possible to distinguish the two particles, since they showed different degrees of contrast against the background The four samples (out of 80) which were wrongly classified by electron microscopy of coded samples were all diluted whole serum aliquots obtained from one individual. HB_sAg could not be demonstrated in this serum sample by either double diffusion or radioimmunoassay (the latter test kindly performed by Dr *Peter Skinhøj* Copenhagen). Nor could HB_sAg associated particles be found in the lipoprotein free fraction of this serum. Structures closely resembling tubular and Dane particles associated with HB_sAg have been observed in sera which are immunologically negative for HB_sAg (9 14 15)

A flattening of LDL molecules on the supporting film may explain the different negative staining properties of the two particles. The amount of stain adhering to a given structure depends – at least partially – on the height of the structure. Thus, the reduced particle height of LDL, due to a proposed flattening may be responsible for the low contrast surrounding these molecules The artificial increase in viral diameter caused by flattening during preparation for electron microscopy has been observed (1 13). The possibility of a corresponding flattening of LDL molecules has been mentioned by *Forte et al* (6) The polygonal appearance of these molecules in areas in which lipoprotein particles are in contact (7 17) also suggests a flexible structure of LDL molecules.

The addition of specific antiserum to preparations of LDL molecules and HB_sAg associated particles caused a significant decrease in the mean diameter of only the former particles. The antibody molecules

may contribute to the retaining of the structure of LDL particles. The fact that the reduction in mean diameter was statistically significant only for the LDL molecules supports the assumption of a more flexible structure of these molecules than of the small HB_sAg associated particles.

Thus, structures which closely resemble each other may be distinguished by careful inspection of negatively stained preparations. The distinction between HB_sAg associated 20 nm particles and LDL molecules was based on the sharper contrast against the background of the HB_sAg particles than that of the native LDL molecules. Furthermore, particle diameters based on measurement of negatively stained preparations may be larger than the native value if flattening of the particles occurs.

My thanks are due to Dr *Peter Skinhøj*, Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, for performing the radioimmunoassay test of one sample. The electron microscopes belong to the Electron Microscopical Unit for Biological Sciences, University of Oslo, Oslo (Head: Dr. *Trond Berg*).

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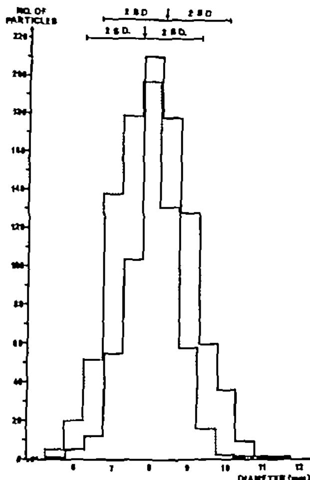


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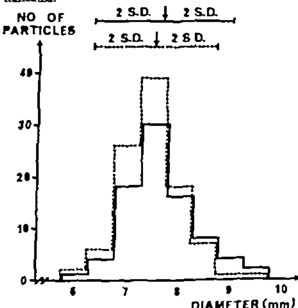


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In spite of these resemblances, it was possible to distinguish the two particles, since they showed different degrees of contrast against the background The four samples (out of 80) which were wrongly classified by electron microscopy of coded samples were all diluted whole serum aliquots obtained from one individual. HB_sAg could not be demonstrated in this serum sample by either double diffusion or radioimmunoassay (the latter test kindly performed by Dr *Peter Skinhøj* Copenhagen) Nor could HB_sAg associated particles be found in the lipoprotein-free fraction of this serum. Structures closely resembling tubular and Dane particles associated with HB_sAg have been observed in sera which are immunologically negative for HB_sAg (9 14 15)

A flattening of LDL molecules on the supporting film may explain the different negative staining properties of the two particles. The amount of stain adhering to a given structure depends – at least partially – on the height of the structure. Thus, the reduced particle height of LDL, due to a proposed flattening, may be responsible for the low contrast surrounding these molecules. The artificial increase in viral diameter caused by flattening during preparation for electron microscopy has been observed (1 13). The possibility of a corresponding flattening of LDL molecules has been mentioned by *Forte et al* (6). The polygonal appearance of these molecules in areas in which lipoprotein particles are in contact (7 17) also suggests a flexible structure of LDL molecules.

The addition of specific antiserum to preparations of LDL molecules and HB_sAg associated particles caused a significant decrease in the mean diameter of only the former particles. The antibody molecules

may contribute to the retaining of the structure of LDL particles. The fact that the reduction in mean diameter was statistically significant only for the LDL molecules supports the assumption of a more flexible structure of these molecules than of the small HB_sAg associated particles

Thus, structures which closely resemble each other may be distinguished by careful inspection of negatively stained preparations The distinction between HB_sAg associated 20 nm particles and LDL molecules was based on the sharper contrast against the background of the HB_sAg particles than that of the native LDL molecules. Furthermore, particle diameters based on measurement of negatively stained preparations may be larger than the native value if flattening of the particles occurs

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My thanks are due to Dr *Peter Skinhøj* Department of Clinical Chemistry Bispebjerg Hospital, Copenhagen, for performing the radioimmunoassay test of one sample The electron microscopes belong to the Electron Microscopical Unit for Biological Sciences, University of Oslo, Oslo (Head Dr *Trond Brdtten*)

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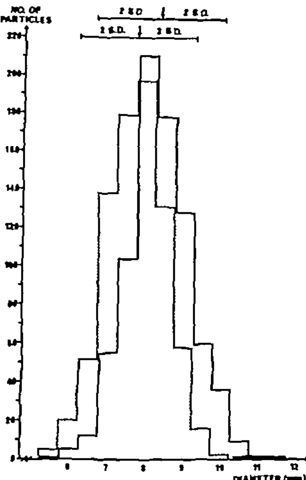


Fig 2 Distributions according to diameter of native *LDL* molecules (*full line*) and *LDL molecules aggregated* after addition of specific antiserum (dotted line) The measurements were made on electron micrographs (magnification × 390 000). Mean diameters (arrows) ± 2 S. D (21 4 nm ± 4 4 nm and 19 9 nm ± 4 0 nm) are indicated

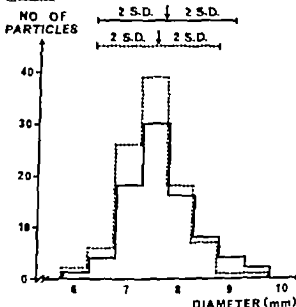


Fig 3 Distributions of free (full line) and antibody aggregated (dotted line) small spherical HB_sAg associated particles by diameter (magnification × 360 000) Mean diameters (arrows) ± 2 S D (?1 2 nm ± 3 8 nm and 20 8 nm ± 3 2 nm) are indicated

of 100 aggregated HB_sAg associated particles was 20 8 nm ± 1 6 nm (Fig. 3). The reduction in diameter observed after addition of antiserum (1 9 per cent) was not significant (t = 1 785 d f = 181 0 05 < p < 0 10).

DISCUSSION

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IMMUNOCHEMICAL STUDIES ON *STAPHYLOCOCCUS AUREUS* PLASMA MEMBRANE

I Isolation and Chemical Characterization

PER AASJORD and ARNE GROV

The Gade Institute, Department of Microbiology University of Bergen, Bergen, Norway

Aasjord, P & Grov A Immunochemical studies on *Staphylococcus aureus* plasma membrane I Isolation and chemical characterization Acta path microbiol scand Sect B, 86 131–137 1978

Cytoplasma membrane and lipoteichoic acid (LTA) were isolated from *S aureus* Cowan I and analysed chemically Pure membrane was obtained by using human IgG coupled to Sepharose, which then absorbed all cell wall fragments due to the interaction between IgG and protein A on the wall LTA, shown to be a glucosylglycerol teichoic acid containing ester-linked alanine and pentadecanoic acid as the major fatty acid was also present in the isolated membrane but only as a minor component Other carbohydrate, protein and lipid components which were present as a chemical complex, dominated the membrane preparations

Key words Staphylococcal cytoplasma membrane, isolation, chemistry

A Grov Mikrobiologisk avdeling, MFH-bygget N-5016 Haukeland sykehus, Norway

Received 27 77 Accepted 3 78

The literature concerning the immunochemistry of the cytoplasma membrane of *Staphylococcus aureus* seems very sparse Except for the membrane-bound lipoteichoic acid (LTA) (5 11 25 26), studies of antigenicity and possible determinants of other constituents have not been published Since the cell wall and cytoplasma membrane are closely connected – and an absolute separation may be difficult to obtain – the importance of a better understanding of the immunochemistry of the membrane is obvious This has particular actuality after the finding that certain membrane structures, e g LTA (28), may penetrate the cell wall This naturally raises the question are LTA or other membrane structures of importance to the antigenicity of the cell?

In the present paper the isolation and the chemical characterization of cytoplasma membrane and LTA is described LTA is isolated to serve as a marker for known structures and for antigenic determinant(s) The serological studies will be reported in a subsequent paper

MATERIALS AND METHODS

Strain

Staphylococcus aureus strain Cowan I (NCTC 8530) was used for the preparation of both plasma membrane and lipoteichoic acid (LTA)

Growth Media

Nutrient broth contained 15 g proteose peptone (Oxoid, England), 2 5 g liver digest (Oxoid), 5 g yeast extract (Oxoid), and 5 g NaCl per 1000 ml Before autoclaving, the pH of the medium was first adjusted to 9 0 with NaOH and then readjusted to pH 7 4–7 6 with HCl, and any precipitate removed by filtration

Blood agar contained 7 5 per cent blood, 1 2 per cent agar (Oxoid), and nutrient broth

Protoplast medium contained 1 6 M sucrose 0 25 per cent $MgSO_4$ and 1000 I U of benzylpenicillin (Apothekernes Laboratorium, Norway) in nutrient broth Penicillin was added just before use

Buffers

Phosphate buffer 0 01 M KH_2PO_4–Na_2HPO_4, pH 6 5 0 25 per cent $MgSO_4$ 0 0 per cent azide

Phosphate buffered saline (PBS) 1/15 M KH_2PO_4–Na_2HPO_4, pH 7 2, 0 85 per cent NaCl

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Immunoadsorbent

Human IgG (AB KABI Sweden) was coupled to cyanogenbromideactivated Sepharose 4B (Pharmacia, Sweden) as described in (20).

Isolation of Cytoplasma Membrane

The blood agar culture (18 h at 37 C) of *S aureus* strain Cowan I was added to the nutrient broth (50 ml) and incubated at 37° C during continuous shaking (160 rot./min) for 5 h then transferred to 400 ml of medium and the same incubation continued for 3 h The bacteria in 130 ml of this culture were harvested by centrifugation (10 min at 8 000 rev/min) suspended in 3 ml of nutrient broth transferred to 400 ml of the penicillin medium, and incubated at 37 C for 36 h during continuous shaking (120 rot /min) The protoplasts were then harvested by centrifugation for 1 h at 20 000 × g The protoplasts, floated to the top were lysed by transfer to 100 ml of phosphate buffer DNase (1 mg, Sigma *DN 100*) and RNase (1 mg Sigma III A) was added to the suspension of lysed protoplasts and the mixture was incubated for 30 min at room temperature. After centrifugation (35 000 × g for 5 min) the pellet formed was resuspended in phosphate buffer to an optical density (OD) of 1 5 at 280 nm and then centrifuged in a discontinuous sucrose gradient (35 to 50 per cent sucrose in phosphate buffer from top to bottom, 5 per cent increase per 5 ml) The protoplast suspension (25 ml) was layered on the top of each tube (100 ml), and the centrifugation was carried out in a swing-out rotor at 1500 × g for 90 min The upper part of the gradient containing the cytoplasma membrane was taken off and centrifuged for 20 min at 35 000 × g the pellet was resuspended to OD1 5 (280 nm) with phosphate buffer and recentrifuged (30 min) on sucrose gradient as before The top layers with membrane fragments were pooled and centrifuged for 20 min at 35 000 × g the pellet then resuspended (extracted) in phosphate buffer and centrifuged for 10 min at 2,000 × g This latter extraction procedure was repeated 3 to 4 times with the precipitate until the supernatant was no longer turbid The membrane material was then collected by centrifugation of the pooled supernatants for 10 min at 20 000 × g All the above centrifugations were carried out at 4 C The plasma membrane material thus obtained was suspended in PBS mixed with Sepharose IgG and incubated with occasional stirring for 1 h at 37° C During this process, IgG is known to fix protein A (8) and thus bind cell wall fragments After sedimentation of the Sepharose particles, the supernatant was taken off and the Sepharose washed once by gentle resuspension in PBS The supernatant and the washing medium were centrifuged for 10 min at 20 000 g the precipitate was then washed 3 times by suspension in distilled water followed by 10 min centrifugation at 20 000 × g and finally freeze-dried

Isolation of LTA

The procedure used was essentially that of *Moskowitz* (15) and *Ofek et al* (17) *S aureus* strain Cowan I (18 h blood agar culture) was incubated 8 h in 100 ml nutrient broth and then transferred to 1000 ml nutrient broth in 2000 ml flasks. All incubations were at 37° C and with continuous shaking at 160 rot./min. The bacteria were harvested after 18 h washed 3 times in distilled water resuspended in 0 2 N HCl (10 ml per g of wet bacteria) and kept in a boiling water bath for 10 min After centrifugation and washing (6 times in cold distilled water), the bacteria were resuspended in distilled water (? ml per g of bacteria), mixed with an equal volume of 95 per cent phenol incubated with vibration (Vibro Mischer Type 1 Bopp & Reuther BRD) for 30 min at 4 C and centrifuged (30 min at 1000 × g) The water phase was thoroughly dialysed and freeze-dried. The LTA material was further subjected to iso-electric focusing (17) using an ampholine-solution with a pH interval of 3 to 6 (LKB Produkter Sweden), and an LKB apparatus with a 110 ml column Finally the LTA preparation was dialysed and freeze-dried

Analytical Methods

Electron microscopic studies of protoplasts and plasma membranes were carried out essentially according to the procedure of *Kellenberger et al.* (10), using 3 per cent glutaraldehyde in 0 2 M cacodylate buffer pH 7 4 (22), as fixative and the Spurr medium (23) for embedding. In the case of protoplasts sucrose was added to the glutaraldehyde-cacodylate buffer to 1 6 M concentration Embedded preparations were cut into thin sections (400 to 500 Å) on an ultramicrotome (LKB Produkter Sweden) and the thin sections were transferred to grids (100 mesh) The grids were treated with 2 per cent aqueous uranylacetate for 20 min, thoroughly rinsed in water and then treated with Reynolds lead citrate for 15 min and washed in water

Electron microscopic studies were carried out in a Hitachi HU 12A electron microscope at 60 KV

Hydrolysis Samples (1 to 2 mg) were hydrolysed in 0 5 ml volumes as follows

(a) 0 1 N HCl for 2 h at 100° C, (b) 3 N HCl for 3 h at 100° C, (c) 6 N HCl for 18 h at 105 C and (d) 1 N NH_3 for 5 min at 100° C (examination for ester-linked amino acids) The hydrolyses were carried out in sealed tubes flushed with nitrogen

Chromatography Circular paper chromatography was carried out on Whatman No 1 paper with the following solvent systems

- A Isopropanol 2 N HCl (65 35 v/v) (12)
- B Propanol NH_3 H_2O (6 3 1 v/v) (1)
- C Butanol HAc H_2O (4 1 1 v/v) (19)
- D Ethylacetate pyridine H_2O (40 11 6 v/v) (7)

The detecting reagents used were ninhydrin (13), the Elson Morgan reagent (18), alkaline silver nitrate (13) and sodium periodate-benzidine (3)

Gas-liquid chromatography (GLC) was carried out on a Perkin Elmer 900 gas chromatograph Amino acids were analysed as trifluoroacetylated butyl esters (21) on columns (0 2 × 200 cm) of Chromosorb W AW HT 80 to 100 mesh covered with 0 325 per cent EGA (ethyleneglycoladipate from Regis, USA) The flow rate of the carrier gas (N_2) was 30 ml/min, and the temperature was raised 4 C/min from 100 to 210° C followed by 8

min at 210° C. The standard amino acid solution, AA 5 was purchased from Calbiochem, USA.

Carbohydrates were analysed as trimethylsilylmethylglycoside derivatives, prepared as described by *Chang et al.* (4) using Sylon-BTZ (Supelco, USA), and fatty acids as methyl derivatives, extracted from methanolysed samples by hexane. Both carbohydrate and fatty acid derivatives were examined on columns of Chromosorb W (80 to 100 mesh), coated with 3 per cent SE 30 (Serva, BRD). Separation of methylated fatty acids, including standards, Mix D and Mix F (Supelco), was obtained by a temperature rise of 4° C/min from 150 to 250° C, and the derivatives of sugars and sugar alcohols were chromatographed by raising the temperature 4° C/min from 100 to 200° C. The flow rate of the carrier gas (N_2) was 30 ml/min. Glycerol was also chromatographed isothermically at 120° C. Standard sugars and sugar alcohols were obtained from British Drug Houses Ltd.

Phosphorus was determined as described in (29).

Serological Tests

Double diffusion in agar was performed as described in (9) using antiserum to *S. aureus* strain Cowan I produced by the intravenous injection of formalin-killed bacteria in rabbits (16), and by using purified cell wall antigens as references.

RESULTS

Variable amounts of inoculum in the penicillin medium resulted in a variable yield of protoplasts, the optimal ratio penicillin medium/inoculum was approximately 400/130. The protoplasts gradually increased in size and finally lysed. After 36 h about 40 per cent of the cells in the culture were protoplasts (Fig. 1). Maximal yield (15 to 20 mg freeze-dried cytoplasma membrane per 1000 ml of medium) was obtained at a penicillin concentration of 1000 I.U./ml, higher concentrations were bactericidal.

The plasma membrane, isolated by sucrose gradient centrifugation in two steps followed by extraction of a sediment, still contained some cell wall fragments detected by double diffusion in agar. These fragments were effectively removed by absorption with Sepharose-IgG. The capacity was by scanning electron microscopy estimated to be 100 to 200 *S. aureus* cells/Sepharose particle (Fig. 3). The final plasma membrane preparation seemed relatively pure according to electron microscopy (Fig. 2), and neither of the cell wall components, *i.e.*

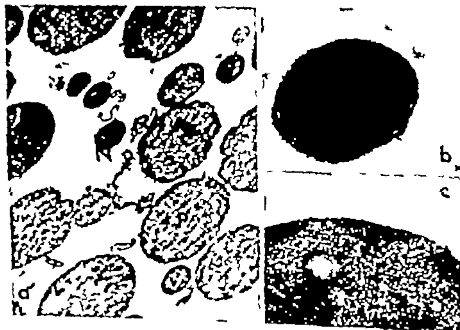


Fig. 1. a. Electron micrograph of the upside layer after flotation showing protoplasts (P) and normal *S. aureus* cells (N) 8,000 ×

b. Higher magnification of a section of Fig. 1a showing a normal *S. aureus* cell. 30,000 ×

c. Higher magnification of a section of Fig. 1a showing a part of a protoplast of *S. aureus* (cf. Fig. 1b). 30,000 ×



Fig 2 a. Electron micrograph of lysed protoplasts of *S aureus* 8 000 ×
b Higher magnification of a section through a vesicle from a lysed protoplast specimen Note the unit membrane nature of the limiting membrane. 80 000 ×

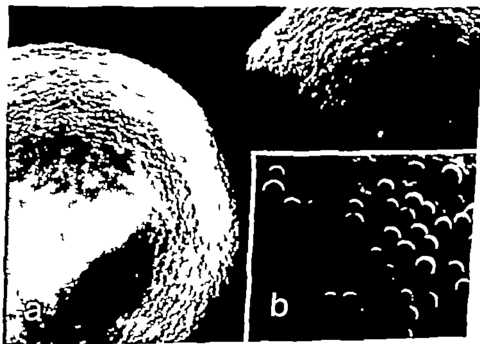


Fig 3 Scanning electron micrographs of *S aureus* Cowan I cells adsorbed onto the surface of a Sepharose gel which was coupled human IgG a 2 500 × b 10 000 ×

teichoic acid and protein A, could be demonstrated serologically. Nor could ribitol from cell wall teichoic acid be demonstrated chromatographically.

The yield of LTA was 4 to 6 mg LTA per 10 g of bacteria (wet weight). On iso-electric focusing, the LTA behaved heterogeneously with serologically active material being observed over a wide pH range.

Chemical Analyses

Paper chromatography of hydrolysates (a) in solvent system A showed no nucleic acid derivatives upon inspection under a UV-lamp. Examination of hydrolysates (b) in system B, C and D revealed the presence of glycerol, glucose, galactose, mannose, and glucosamine in the cytoplasma membrane and glycerol and glucose in LTA. These findings were verified and the substances quantitated by gas chromatography (Table 1). Concerning glycerol determination, prehydrolysation with 3N HCl for 3 h at 100° C before esterification gave higher values.

The result of gas chromatographic examination of fatty acids present in the membrane and LTA preparations is shown in Table 2. The samples were prehydrolysed (3 N HCl, 3 h at 100° C) before esterification. C 15 (pentadecanoic acid) was the major fatty acid found both in membrane and LTA, counting for about 75 per cent of total fatty acids observed in the latter.

The amino acid composition of cytoplasma membrane and LTA is given in Table 3. The cytoplasma membrane was found to be richer in amino acids than LTA, both qualitatively and quantitatively, with the exception of alanine, the major amino acid of LTA. Alanine was also released from both membrane and LTA upon digestion in 1 N NH_3 for 5 min at 100° C, indicating the presence of ester-linked alanine.

The total amounts of phosphorus in membrane and LTA preparations were estimated to 0.238 µmol/mg and 0.428 µmol/mg, respectively.

TABLE 3. *GLC Analyses of Amino Acids[a] in Cytoplasma Membrane and LTA of S. aureus Cowan I*

Amino acids	Membrane µmol/mg	LTA µmol/mg
Ala	0.106	0.158
Val	0.068	0.040
Gly	0.066	0.033
Ileu	0.073	0.037
Leu	0.143	0.063
Pro	0.054	0.016
Thr	0.067	0.029
Ser	0.049	0.057
Phe	0.120	0.040
Asp	0.238	0.102
Glu	0.292	0.128
Tyr	0.055	0.–
Orn	0.031	0.–
Lys	0.194	0.113
Trp	0.100	0.–

a) The figures are the mean of three individual samples (hydrolyses).

TABLE 1. *GLC Analyses of Sugars and Sugar Alcohols in Cytoplasma Membrane and LTA of S. aureus Cowan I*

	Membrane µmol/mg	LTA µmol/mg
Glucose	0.87	0.167
Galactose	0.55	–
Mannose	0.30	–
Glucosamine	0.44	–
Glycerol	0.543	0.760

TABLE [illegible]. *GLC Analyses of Fatty Acids[a] in Cytoplasma Membrane and LTA of S. aureus Cowan I*

Fatty acids	Membrane µmol/mg	LTA µmol/mg
C 14:1[b]	0.011	–
C 15 (Pentadecanoic acid)	0.257	0.256
C 16:1	0.008	–
C 16 (Hexadecanoic acid)	0.0[illegible]4	0.013
C 17:1	0.059	0.037
C 18 (Octadecanoic acid)	0.064	0.021
C 19:1	0.021	–
C 20 (Eicosanoic acid)	0.093	0.005
C [illegible] (Docosanoic acid)	0.018	–

a) Figures are the mean of two parallel determinations.
b) Location of the double bond is unknown.

DISCUSSION

The yield of protoplasts is apparently dependent on the amount of bacteria added to the penicillin-containing medium, and the optimal ratio of inoculum/protoplast medium has to be adjusted. High speed centrifugation of protoplasts in a medium containing sucrose results in a flotation of protoplasts and thereby a considerable concentration as well as purification. Thus, as shown by microscopy (phase contrast and electron), about 70 per cent of the cells in the upper layer were



Fig 2 a. Electron micrograph of lysed protoplasts of *S. aureus*. 8 000 ×
b Higher magnification of a section through a vesicle from a lysed protoplast specimen. Note the unit membrane nature of the limiting membrane. 80 000 ×

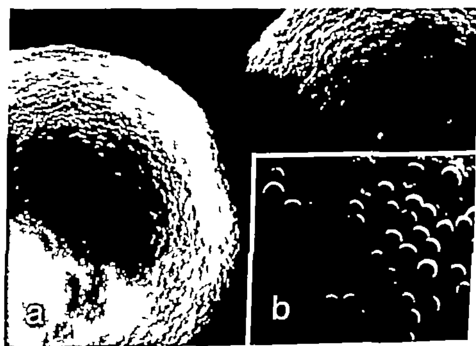


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[illegible] der Papierchromatographie. E. Merck AG, Darmstadt.

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protoplasts. Suspension in disodium-phosphate buf fer resulted in small membrane fragments, whereas sodium-potassium-phosphate buffer resulted in nearly intact membranes. The yield of purified membrane was rather low but based on chemical and serological criteria, it was free of cell wall constituents. This is very important and the high degree of purity was easily obtained by utilizing the affinity between human IgG and staphylococcal protein A The method may also have wider application if antibodies to specific cell wall constituents covalently coupled to Sepharose or other inert particles are used. Morphologically ribosomes cannot be excluded, but chemically no nucleic acid component was detected.

Lipoteichoic acid (LTA) is a component of the cytoplasma membrane and LTA from several bacteria has been characterized both chemically and serologically Thus, in these experiments, LTA was used as a reliable marker for both chemical constituents and for antigenic determinants. LTA has been found to be a glycerolphosphate polymer with glycosyl and alanyl substituents and a lipid moiety (26) In the LTA of *S aureus* Cowan I there was a glucosyl substituent and the release of alanine after a short exposure to 1 N NH_3 indicated the presence of ester linked alanine as well. This amino acid was the major one found in LTA. The major fatty acid present was C 15 in agreement with the findings of *Asselineau*(2) who reported 45 per cent of C 15 in *S aureus* Fatty acids apparently vary from bacteria to bacteria (27) The ratio glycerol/glucose in LTA of strain Cowan I was 1 0·0 22 whereas in strain H this ratio was estimated at 1 0·0 66 (6), indicating a 3 times higher glucosidic substitution in the latter strain. The phosphorus glucose alanine ratio in *S aureus* Cowan I was 1 0·0 39·0 37 In group N streptococci the corresponding ratio was estimated at 1 0·0 12·0 14 (27) The glucose: alanine ratio seems similar but the degree of substitution is apparently 3 times higher in *S aureus* Cowan I than in group N streptococci. Assuming a 1 0 1 0 ratio of phosphorus and glycerol in teichoic acid about half the amount of glycerol found in LTA belongs to the lipid component. The LTA preparation must be considered sufficiently pure for the present investigation even though some other amino acids were present, and a heterogeneity in charge was observed by electrophoretic focusing In addition to the components of LTA, the membrane preparations of *S aureus* Cowan I contained other sugars, amino acids and fatty acids in amounts indicating carbohydrate, protein and lipid structures quantitatively of greater importance than the LTA. Most probably a considerable part of the LTA is extracted during isolation and purification of the membrane. The total amount of protein of strain Cowan I cytoplasma membrane was 23 per cent, slightly more than half the amount of *S aureus* cytoplasm membrane given by *Mitchell* (14).

Theodore & Panos (24) found that the amount of fatty acids in *S aureus* cytoplasma membrane was 20 per cent which agrees fairly well with the present results, i.e 17.5 per cent. As in LTA the main fatty acid was pentadecanoic acid.

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IMMUNOCHEMICAL STUDIES ON *STAPHYLOCOCCUS AUREUS* PLASMA MEMBRANE

2. *Antigenic Properties*

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Aasjord, P. & Grov, A. Immunochemical studies on Staphylococcus aureus plasma membrane. 2. Antigenic properties. Acta path. microbiol. scand. Sect. B, 86: 139–141, 1978.

Cytoplasmic membrane and lipoteichoic acid (LTA) isolated from *S. aureus* Cowan I were examined serologically. LTA contains both α- and β-glucosyl substituents at glycerol and most probably ester linked alanine as well, all being antigenic determinants. In addition to LTA, the membrane contains a glycoprotein exhibiting antigenic determinant(s) in both the protein and sugar moieties.

Key words: Staphylococcal cytoplasmic membrane; antigenic determinants.

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In a previous paper (10) the cytoplasmic membrane and lipoteichoic acid (LTA) were isolated from *Staphylococcus aureus* strain Cowan I and analyzed chemically. Both chemically and serologically LTA was shown to be part of the plasma membrane. This membrane also contained other lipid components as well as glycoprotein(s).

The present paper reports the results of further serological examinations and a tentative characterization of the antigenic determinants.

MATERIALS AND METHODS

Antigens

The cytoplasmic membrane and lipoteichoic acid (LTA) preparations used were those described previously (10). Poly A_3 from *S. aureus* Wood 46 (3), poly B_4 from *S. epidermidis 1254* (6), poly B_3 from *S. epidermidis* T_1 (9), protein A and protein B (1), and peptidoglycan were included as references.

Sera

Antisera to whole bacteria were raised in New Zealand white rabbits of the Institute's breed by intravenous injections (7). Cytoplasmic membrane and LTA, mixed with Freund's complete adjuvant (Difco, USA), were first injected into one of the rabbit's hind foot pads followed by intramuscular injections, 14 days apart, of membrane/LTA in Freund's incomplete adjuvant. Each dose contained 2 mg of antigen and each rabbit received a total of 12 to 18 mg. The rabbits were bled one week after the last injection, and the sera were inactivated by incubation for 30 min at 56°C, and 1 drop of 1 per cent merthiolate per 5 ml was added as preservative.

Trypsin Treatment

Samples (0.2 mg in 0.5 ml of phosphate buffered saline (PBS), pH 7.4) of plasma membrane and LTA were incubated with 0.1 per cent trypsin (Type XI, Sigma, USA) at 37°C overnight. The trypsin activity was stopped by an equivalent amount of trypsin inhibitor (Type 1-S, Sigma).

Periodate Treatment

Samples (0.4 mg) of plasma membrane and LTA were incubated with 1 per cent sodium periodate (Merck, BRD) in water (1 ml) for 18 H in the dark at room temperature followed by dialysis against PBS.

Serological Tests

Double diffusion in agar was performed as described in (4), and *indirect haemagglutination*, using both normal (NSE) and tanned (TSE) sheep erythrocytes was carried out as described in (8) using a sensitizing dose of 0.2 mg of antigen per 10 ml of 0.5 per cent blood. *Inhibition of*

LTA by both α- and β-glucosylglycerol teichoic acids indicates that teichoic acid determinants are exposed on NSE. Secondly the results also show that both sugar anomers probably are present. The inhibition by poly B_a and poly B_j could be due to the blocking of anti-glycerolphosphate antibodies, but in that case, providing equal substitution, the inhibitory effect should be the same (same titre) The glucosylglycerol teichoic acids did not inhibit agglutination of sensitized TSE. Apparently these determinants are blocked on TSE, or another dominating determinant(s) is revealed. In this case, a possible determinant may be the ester-linked alanine (D-alanine). The low titres in all sera using sensitized NSE may indicate that (a) very few of the teichoic acid determinants are exposed, (b) that relatively few antibodies to the free determinant are present, or (c) that these antibodies are bad agglutinins. The high titres obtained using sensitized TSE may either indicate strong agglutinins or indicate that the titre gives the sum of several determinants

According to the chemical and serological examinations, the cytoplasma membrane is composed of LTA and a glycoprotein, both components being immunogenic and exhibiting several antigenic determinants LTA apparently contains both α- and β-glucosyl substituents It is also strongly indicated that ester-linked alanine is an antigenic determinant The glycoprotein exhibits antigenic determinant(s) both in the protein and sugar moieties None of the determinants seems to have any relation to cell wall antigens

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Indirect haemagglutination was carried out as in (2) using 4 agglutinating units of the antisera. *Absorption of antigen* (0 2 mg samples) with NSE and TSE was continued till the cells used for absorption no longer *agglutinated in a potent antiserum.* The supernatants were then dialysed, freeze-dried and tested for activity on double diffusion and indirect haemagglutination.

RESULTS

Double Diffusion in Agar

Cytoplasma membrane gave two lines against *homologous* sera, one of the *lines* being confluent with the single line of LTA. Thus, the common *component* was verified serologically Antisera against purified membrane or LTA gave no reaction *with any of the reference antigens. However* an antiserum to cytoplasma membrane not absorbed with Sepharose-IgG (10), *did react with protein A* and B Reaction with protein B showed that specific antibodies were formed This again is a clear-cut demonstration of the purification effect of the absorption step (10) Periodate treatment of plasma membrane and of LTA destroyed the precipitating ability of both preparations Trypsination removed one of the lines given by native cytoplasma membrane, whereas the LTA-line and the corresponding line formed by the membrane remained intact. Absorption of membrane material and LTA by NSE removed all precipitating activity

Indirect Haemagglutination

NSE sensitized with cytoplasma membrane agglutinated in homologous sera to a titre of 1/40 whereas TSE agglutinated to 1/2560 NSE and TSE *sensitized with LTA agglutinated in* homologous sera to titres of 1/160 and 1/5120 respectively The two *antigens and antisera gave cross* reactions with both types of blood cells.

Both poly B and poly B_β inhibited agglutination of NSE sensitized with LTA in anti LTA sera, the inhibiting doses were 1/8 and 1/128 of 1 mg per ml respectively No other haemagglutination systems (NSE or TSE sensitized with cytoplasma membrane in anti-cytoplasma membrane sera or TSE LTA in anti LTA) were inhibited by poly $B_{\alpha,\beta}$. Nor were any of the other single reference antigens or peptidoglycan effective as inhibitors in concentrations up to 5 mg/ml

After trypsination of cytoplasma membrane, the *titre of sensitized NSE remained* unchanged whereas the titre of sensitized TSE went down from 1/2560 *to* 1/80 The same effect was observed with trypsinated LTA The titre of sensitized NSE remained unchanged whereas the titre of TSE went down from 1/5120 to 1/320 No activity *could be* observed after treatment of membrane and LTA with sodium periodate.

TSE sensitized with supernatants after absorption of cytoplasma membrane and LTA with NSE did not agglutinate, indicating that NSE absorb all antigenic material.

DISCUSSION

Double diffusion in agar confirmed the results of the chemical examinations (10), which showed the presence of LTA in the cytoplasma membrane. Thus, common properties between the two preparations must be due to LTA. The lack of observable antibodies to *S aureus* cell wall antigens in specific antisera strengthens the criteria for purity Resistance to trypsin and sensitivity to periodate regarding the precipitating ability of LTA is in accord *with previous findings that glucosylglycerol teichoic* acid is the antigenic determinant (5). Although the electrophoretic experiments indicated heterogeneity of LTA, both double diffusion in agar and absorption to sheep cells showed that LTA is one antigenic entity

The cytoplasma membrane apparently contained one additional precipitinogen. Being both trypsin and periodate sensitive, this antigen probably is a glycoprotein in which both intact protein and sugar moieties are necessary for precipitation. Glucose was the only sugar present in LTA, whereas the other sugars demonstrated in the cytoplasma membrane (10) are part of the glycoprotein.

The titres of NSE sensitized with cytoplasma membrane and with LTA were unchanged after trypsination of the antigens, thus the sugar determinants were still intact and exposed to antibodies. In contrast, TSE sensitized with trypsinated antigens agglutinated at a much lower titre than with untreated antigens. Since all antigens were absorbed by NSE (and TSE), the above observation implies that most of the antigenic determinants exposed to antibodies on sensitized TSE are trypsin sensitive. No serological activity was observed after periodate treatment. This may indicate that sugars are completely or *partly involved in all determinants, or* that binding sites to erythrocytes are dependent on periodate sensitive structures Most probably different sites on the antigens are involved in the linkages to NSE and TSE and consequently various determinants are exposed or available to reaction with antibodies. Since all antigens were absorbed, this is clearly demonstrated by the large difference between the titres of sensitized NSE and TSE of the same serum Also, *the results of inhibition experiments* point to the same conclusion Firstly the agglutination inhibition of NSE sensitized with

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IMMUNOCHEMICAL STUDIES ON THE SPECIFIC AGGLUTINOGENS OF *STAPHYLOCOCCUS AUREUS*

1. Isolation and Characterization of Antigen h_1

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The specific *Staphylococcus aureus* agglutinogen h_1 has been purified and shown to be a protein with a molecular weight of about 95,000. Chemical analyses revealed all the common amino acids, except tyrosine and the sulphur-containing ones. The purified h_1 antigen was strongly immunogenic in rabbits. The antiserum produced one precipitation line on double diffusion in agar against a suspension of bacteria. It also agglutinated bacteria of the h_1-containing strains, as well as tanned sheep erythrocytes sensitized with h_1, in high dilutions. Antibodies to other known staphylococcal antigens were not detected. The identity between h_1 and Pillet's type 9 antigen was confirmed.

Key words: *Staphylococcus aureus*; agglutinogens; immunochemistry.

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Little is known of the chemical nature of the *Staphylococcus aureus* type agglutinogens, although the majority are supposed to be proteins. Isolation of these antigens is difficult, since they appear only in very small amounts in the cell wall. Only one report on the partial characterization of an agglutinogen (*a*) has appeared (8). The scant knowledge of the chemical nature of the specific agglutinogens is an obvious disadvantage in serotyping *S. aureus*.

The aim of the present work was to isolate and characterize the agglutinogen h_1. This antigen was chosen because it produces a precipitation line in agar gel (22). Such might be a convenient indicator during purification. Furthermore, we wanted to test the immunogenicity of the purified antigen to see if a monospecific factor serum could be produced which might replace the absorbed whole serum now in use for serotyping *S. aureus*.

MATERIALS AND METHODS

Strains

S. aureus 17A, belonging to Oeding's type strains (19) and known to react strongly with h_1 factor serum, was chosen for the production of the h_1 antigen. Strain 17A has the antigenic pattern $a_3/h_1/h_2/263\text{–}2$ (9, 13). For comparison, antigen 9 was isolated from Pillet's type strain CK9 (23). This antigen has been reported to be identical to the h_1 antigen (4). Other type strains were included as controls according to their content of antigen h_1 (strain 670), or to the absence of h_1 but presence of one of the other antigens contained in strain 17A, viz. antigen a_3, h_2 and 263–2 (strains 830, 5687, 263, Cowan I, 3647).

Cultivation

The bacteria used for the isolation of antigen were grown in nutrient broth, containing 15 g proteose peptone (Oxoid, England), 2.5 g liver digest (Oxoid), 5 g

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Cultivation

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were mixed with an equal volume of antigen in saline and incubated at 37° C for 30 min. *Ring test* precipitation was performed as described in (11) and *double diffusion in agar* as in (12). *Indirect haemagglutination* using normal (NSE) and tanned sheep erythrocytes (TSE) was performed as before (21). The amount of h_1 antigen used for sensitization was 0.1 mg per 20 ml of 0.5 per cent sheep red cells.

RESULTS

After four extractions of the 17A bacteria, only traces of h_1 could be detected in the bacterial pellet by the double diffusion test. The h_1 antigen precipitated completely at a pH of between 2.5 and 3 but so did the majority of protein A, as well as protein B.

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TABLE 1. *GLC Analysis of Amino Acid Composition[a] of h_1 from S. aureus*

Amino acids	µmol/mg	Per cent by weight
Ala	0.16	1.4
Val	0.34	4.0
Gly	0.64	4.8
Ileu	0.42	5.5
Leu	0.33	4.3
Pro	0.66	7.6
Thr	0.99	11.8
Ser	0.36	3.8
Phe	0.44	7.2
Asp	0.84	11.2
Tyr	0.14	2.6
Glu	1.20	17.7
Lys	0.97	14.1
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[a] The values given were obtained by linear extrapolation to zero time.

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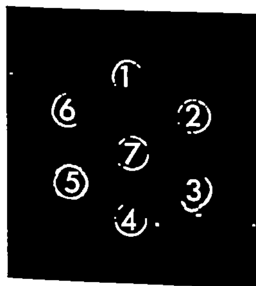


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Hydrolysis. Samples (1–? mg) of antigen were hydrolysed in sealed tubes, flushed with nitrogen, in 0.5 ml volumes of 3 N HCl for 3 h at 100° C and 6 N HCl for 20 h and 72 h at 105° C. The hydrolysates were evaporated to dryness *in vacuo* over NaOH pellets.

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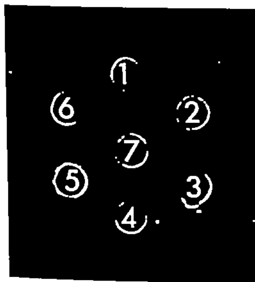


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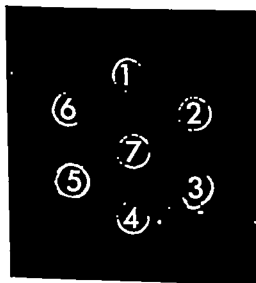


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h_1 is the first of the specific *S. aureus* agglutinogens which has been isolated and characterized. The successful production of a very potent monovalent h_1 antiserum is an important improvement in the serotyping technique. By the traditional technique, factor sera for typing are produced by absorption of whole rabbit sera. The factor sera are often low titred and contain dissolved antigenic material which may disturb agglutination. The present h_1 antiserum can be used in high dilutions, *i.e.* between 1/10,000 and 1/5 000 (2–4 agglutinating units). Provided that also other specific *S. aureus* agglutinogens can be isolated, more standardized procedures for typing of staphylococci will be possible.

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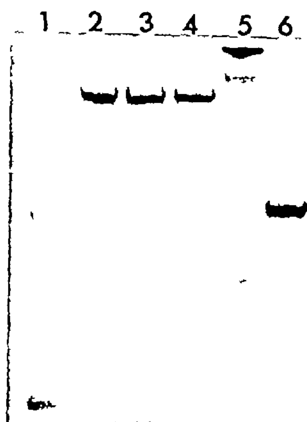


Fig 2 SDS polyacrylamide gel electrophoresis of reduced and alkylated human IgG (1), h_1 (17A) (2) h_1 (CK9) (3), h_1 (17A) unreduced (4), partially reduced human IgG (5), and human serum albumin (6).

of 70° C for 30 min, but labile at 100° C. Trypsinization destroyed the serological activity All the common amino acids except tyrosine and the sulfur-containing ones, were demonstrated (Table 1). Only some slight variations in the molar ratios of amino acids were observed in h_1 preparations from the two staphylococcal strains examined The major amino acids were Glu, Lys, Asp, Thr Pro and Phe. On SDS electrophoresis only one band was observed, also after exposure to 0 2 M 2 mercaptoethanol (Fig 2) The distance moved, compared to the reference proteins, indicated a molecular weight of 95 000 Immunoelectrophoresis showed a single line using 17A whole serum There was no electrophoretic movement of h_1 at pH 8 6

The h_1 preparations tested were all immunogenic in rabbits. In double diffusion in agar the antisera obtained gave only one line against purified h_1 as well as against 17A bacteria (Fig 1). In one antiserum, 17A bacteria agglutinated at a dilution of 1/18 000 The 670 bacteria agglutinated at a dilution of 1/16 000 whereas all the h_1 negative strains tested, except strain Cowan I did not agglutinate at a dilution of 1/10 This also demonstrates that h_1 is free of other known antigens of strain 17A The reason why the titre of Cowan I agglutination increased from 1/100 of preimmune serum to 1/800 is obscure, but it is a usual observation by immunization of staphylococci or isolated compounds. However the increased titre of Cowan I agglutination did not influence the h_1 tests. Five blood samples, taken 10 days apart after the first injection, and treated with 2 mercaptoethanol, showed the same titres as untreated samples. Thus, little or no IgM activity seems to be involved in the bacterial agglutination test.

There was no agglutination of NSE treated with the h_1 antigen, whereas TSE sensitized with h_1 agglutinated in the h_1 antiserum mentioned above at a dilution of 1/18 000 and in h_1 factor serum at a dilution of 1/80 Thus, there is an apparent correspondence between antibodies participating in bacterial agglutination and indirect haemagglutination.

DISCUSSION

Antigen h_1 has been shown to be a heat-labile and trypsin-sensitive protein having a molecular weight of approximately 95 000 The present investigation confirms earlier indications (4) that Pillet s antigen 9 is identical to h_1 Apparently the h_1 antigen is firmly bound to the cell wall. The release, probably caused by autolytic activity was slow and required both a long incubation time and a change of buffer By using the cell wall lytic enzyme lysostaphin (14), a more rapid release of h_1 was observed. This may indicate that h_1 is bound to the peptidoglycan structure. The amount of h_1 in the cell is apparently low like most agglutinogens. Compared to the yield normally obtained for teichoic acids (polysaccharides) h_1 comprises only about one tenth of that weight (6) Neither the wall teichoic acid nor the a_5 agglutinogen are precipitated by acid and were both removed at the first fractionation. Protein A (and protein B) was completely removed by the immunoadsorbent, whereas the stage(s) at which h_2 and 263–2 were removed is more uncertain. Both the ion-exchange fractionation and the final gel filtration removed UV-absorbing materials of at least three different molecular sizes

The purity of the h_1 antigen was confirmed in two ways. Firstly h_1 gave a single line on agar diffusion against whole sera, one single band on SDS electrophoresis, and did not inhibit agglutination in factor sera a_5, h_2 and 263–2 Secondly the potent h_1 antisera contained only h_1 antibodies. a_5, h_2 and 263–2 agglutinins could not be detected. The strong immunogenic property of h_1 is most likely a consequence of its relatively high molecular weight, but its high content of the amino acids threonine

and phenylalanine may also be of importance in this context.

h_1 is the first of the specific *S. aureus* agglutinogens which has been isolated and characterized. The successful production of a very potent monovalent h_1 antiserum is an important improvement in the serotyping technique. By the traditional technique, factor sera for typing are produced by absorption of whole rabbit sera. The factor sera are often low titred and contain dissolved antigenic material which may disturb agglutination. The present h_1 antiserum can be used in high dilutions, i.e. between 1/10,000 and 1/5 000 (2–4 agglutinating units). Provided that also other specific *S. aureus* agglutinogens can be isolated, more standardized procedures for typing of staphylococci will be possible.

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IN VITRO EFFECT OF COLCHICINE ON NEUTROPHIL GRANULOCYTE LOCOMOTION

Assessment of the Effect of Colchicine on Chemotaxis, Chemokinesis and Spontaneous Motility using a Modified Reversible Boyden Chamber

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Valerius, N. H. *In vitro* effect of colchicine on neutrophil granulocyte locomotion. Assessment of the effect of colchicine on chemotaxis, chemokinesis and spontaneous motility using a modified reversible Boyden chamber. Acta path. microbiol. scand. Sect. B, 86 149–154 1978.

The effect of colchicine on human neutrophil granulocyte chemotaxis, chemokinesis and spontaneous motility was examined, using a modified reversible Boyden chamber. Colchicine was shown to inhibit the attraction of neutrophils to casein and to a bacterial chemotactic factor at concentrations as low as 10^{-7} M. Experiments in which the absolute concentrations and the concentration gradients of the chemotactic agent were varied, revealed that colchicine inhibited chemokinesis rather than chemotaxis. The spontaneous motility measured in the absence of chemotactic agents was not inhibited by colchicine. Pre-incubation of the cells with a bacterial chemotactic factor did not change the sensitivity of the cells to colchicine. It is concluded that the integrity of microtubule function is not necessary for the ability of the cells to discern a concentration gradient or to react to this with directional locomotion. Thus the inhibitory effect of colchicine on neutrophil granulocyte chemokinesis may not depend on its inhibition of microtubule function. It is suggested that colchicine may block the still unidentified membrane mechanism involved in the translation of the recognition signal into an appropriate locomotory cell response.

Key words: neutrophil granulocyte, chemotaxis, locomotion, colchicine, microtubules.

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Leukocytes may respond to a variety of chemical stimuli with enhanced active migration (22) which may be directional (chemotactic) or random (chemokinetic) (13). A chemotactic response may occur when leukocytes are exposed to a concentration gradient of the chemical stimulus, towards the source of which the cells can then be shown to migrate actively. Chemokinesis is the enhanced non-directional active locomotion observed on exposure of the cells to most chemoattractants in the absence of a concentration gradient. Thus in Boyden chemotaxis assays (8) in which the cells are allowed to migrate through micropore filters towards a chemotactic agent, the activity observed will result from the combined effects of chemotaxis and chemokinesis (21, 22, 23).

The ability of leukocytes to respond to chemical gradients with directional locomotion is believed to require an intact microtubule function (1). Colchicine is an efficient inhibitor of the microtubule system. This agent acts by binding to monomeric tubulin thus preventing its polymerization into microtubules (19). Most previous studies have found antitubulins to be effective inhibitors of leukocyte chemotaxis (2, 3, 4, 6, 7, 9, 11, 18, 20). In one investigation (4) it was also observed that the random movement of granulocytes was affected

only slightly by pre-treatment of the cells with demecolcine, an active derivative of colchicine. However in this latter study the techniques for measuring chemotaxis and random motility were quite different from each other and no attempt was made to distinguish between the effect of demecolcine on chemotaxis and chemokinesis.

It was therefore decided to examine the separate effects of colchicine on neutrophil granulocyte spontaneous motility, chemotaxis and chemokinesis, using a modified Boyden technique in which the concentrations of the chemoattractant were varied above and below the filter in a series of tests. This permits evaluation of the influence of both the concentration gradient of the chemoattractant (chemotaxis) and its absolute concentration (chemokinesis) (21–23).

MATERIALS AND METHODS

The polymorphonuclear leukocyte (PMN) chemotactic activity was determined using a modified reversible Boyden chamber as previously described (21). Briefly, blood leukocytes from normal donors were suspended in Gey's balanced salt solution (GBSS) containing 2% human albumin at a concentration of 1×10^6 PMN per ml after removal of the red cells by dextran sedimentation and hypotonic lysis. The chemotactic agents were casein, dissolved in GBSS at 5 mg per ml, and a bacterial chemotactic factor (BCF), a culture filtrate of *E. coli* diluted with GBSS as indicated. In the upper compartment of the Boyden chamber 5×10^5 PMN were placed, being separated from the chemotactic agent by a 3 micron pore size micropore filter (Sartorius, Gottingen, Germany). The chambers were incubated for 2½ h at 37° C. After incubation for 2 h the chambers were turned upside down in order to prevent cell detachment from the lower surface of the filter. The filters were then fixed and stained with haematoxyline. Counting was made of cells that had migrated completely through the filters and were lying on their attractant surfaces, using an automatic image analysis system (Classimat, Leitz, Wetzlar, Germany). The chemotactic activity was expressed as the mean number of cells per screen field at 10×10 amplification. All experiments were performed in triplicate, and the results represent the median.

The distinction between chemotaxis and chemokinesis was performed using the »checkerboard« assay first described by *Zigmond & Hirsch* (23). This consisted of a series of chambers in which the chemoattractant was placed above the filter in the cell compartment, below the filter or in both compartments at varying concentrations. Each triplicate of samples thus differed from the others as regards the absolute concentration of the attractant, the concentration gradient across the filter or both. Details of the concentrations used are shown in Table 1.

Incubations with colchicine (Sandoz, Basel, Switzerland) at the concentrations indicated were performed for 60 min at 37° C with a cell concentration at 2×10^6 PMN per ml. After this incubation, the cell suspensions were diluted with equal volumes of GBSS containing 4% human albumin before being tested for leukotactic activity in the continued presence of colchicine at half the indicated concentrations. In the experiments using cells pre-incubated with either BCF diluted 1:3 with GBSS or GBSS, this was performed for 30 min at 37° C. After pre-incubation, the cells were washed twice in GBSS before being tested as described above with varying concentrations of colchicine.

All experiments included control samples incubated without colchicine. The inhibition of the activity was determined as

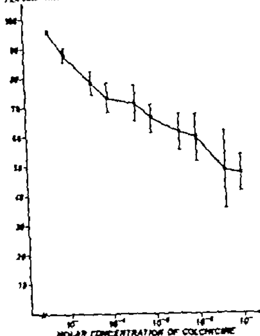
$$100 - \frac{(\text{activity in colchicine-treated samples}) \times 100}{\text{activity in control samples}}$$

Viability of the cells was assured by Trypan Blue exclusion test.

RESULTS

It will be seen from Fig. 1 that pre-incubation of PMN at varying concentrations of colchicine resulted in a dose-dependent inhibition of the leukotactic response of the PMN to both casein and BCF. This inhibition was obvious at concentrations down to 10^{-7} M. A 50% inhibition was observed after incubation with colchicine at a concentration of 10^{-6} M. There was no significant difference in the inhibitory effect of colchicine on attraction with either casein or BCF.

The effect of colchicine at a concentration of 5×10^{-4} M on chemotaxis and chemokinesis induced by BCF can be seen from Table 1. In these experiments the cells were allowed to migrate through the filter in positive, i.e. from lower towards higher concentrations of BCF, and in negative concentration gradients and without gradients at different concentrations of BCF. Along the diagonals from upper left to lower right in the tables, the absolute concentration of the chemotactic factor increased in the absence of a concentration gradient across the filter. The figures on these lines thus indicate the chemokinetic activity. It can be seen that the activity of both colchicine-treated and untreated cells increased with increasing concentrations of BCF. However, the increase was much lower in the colchicine-treated cells than in the untreated cells, thus showing that colchicine has a strong inhibitory effect on chemokinesis. The concentration gradient across the filter was positive above these diagonals and negative below. The higher figures above than below in both colchicine-treated cells and untreated cells indicate a uni-directional locomotion of the cells towards BCF. The influence of the concentration gradient at various absolute concentrations of BCF is seen along the lines parallel to the diagonals



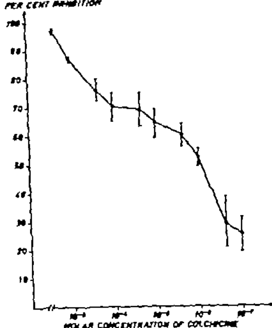


Fig. 1 Dose-dependent inhibition by colchicine of the locomotory response of PMN. Results from attraction with casein (5 mg per ml) (A) and BCF (diluted 1:3 with Gey's solution) (B). Abscissa: Molar concentration of colchicine. Ordinate: Percentage inhibition of activity. Mean ± 1 SEM of four experiments.

from lower left to upper right. Along these lines the concentration gradients reversed from negative to positive, while the absolute concentrations of BCF were kept constant. Although the figures are generally lower in colchicine-treated cells than in untreated cells as the result of inhibited chemokinesis, the proportional increase in the cell numbers along these lines is nearly equal. This shows that the cells had retained their ability to discern and react with directional locomotion to a concentration gradient.

The tables also show that the activity in the complete absence of chemotactic agents was equal in colchicine-treated and untreated cells, thus indicating that the spontaneous motility of PMN is not affected by colchicine at this concentration. The results of using casein as the chemotactic agent are not shown here, but they did not differ from those obtained with BCF.

Fig. 2 shows the dose-dependent inhibition by colchicine of PMN attraction with BCF after pre-incubation of the cells with BCF or GBSS. It can be seen that the inhibition curves are almost identical, thus indicating that pre-incubation of the cells with BCF did not affect the sensitivity of the cells to colchicine.

TABLE 1. *Effect of Varying the Concentration Gradient and the Absolute Concentration of a Bacterial Chemotactic Factor (BCF) on the Locomotory Response of Human Neutrophil Granulocytes, Pre-Incubated with either Gey's Solution (A) or Colchicine at a Concentration of 5 × 10⁻⁶ M (B) for 60 Min at 37° C. Mean of T[illegible] Experiments*

A		Concentration of BCF (%) in Attractant Compartment			
		0	12	20	32
Concentration of BCF (%) in Cell Compartment	0	4	69	117	304
	12	20	79	142	275
	20	[illegible]	72	161	291
	32	18	11	181	246

B		Concentration of BCF (%) in Attractant Compartment			
		0	12	20	32
Concentration of BCF (%) in Cell Compartment	0	5	22	36	61
	12	7	14	27	60
	20	6	18	22	51
	32	6	18	31	58

In all samples tested, the viability of the cells was ensured by Trypan Blue exclusion test showing more than 95% dye-excluding cells

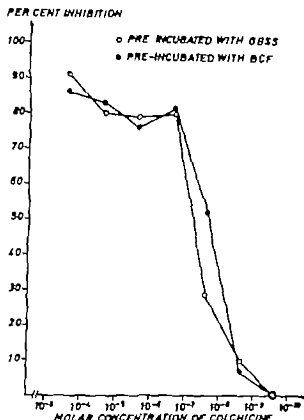


Fig 7 Dose-dependent inhibition by colchicine of the attraction of PMN with BCF (diluted 1 3 with Gey's solution) The cells were pre-incubated either with BCF (diluted 1 3) (closed circles) or with Gey's solution (open circles) Abscissa Molar concentration of colchicine. Ordinate Percentage inhibition of activity Mean of two experiments.

DISCUSSION

It is generally recognized that colchicine affects the microtubular system in the cells by binding to tubulin, thus preventing its polymerization into functional microtubules (19) However colchicine has been shown to affect also certain cell functions which are not supposed to be microtubule dependent such as nucleoside transport across cell membranes (5) and oxygen consumption in phagocytes (14)

Most authors are agreed that colchicine and other antitubulins act as efficient inhibitors of leukocyte chemotaxis *in vitro* (2 3 4 6 7 9 11 18 20) The failure of one study (10) to demonstrate this was probably the result of omitting pre-incubation of the cells with colchicine. The present results shown in Fig. 1 are quite consistent with these previous observations in showing a dose-dependent inhibition by colchicine of the attraction of human PMN by casein and BCF This inhibition was obvious at colchicine concentrations at 10^{-7} M

It was also evident that, even at very high concentrations of colchicine (10^{-3} M) some cells had retained their ability to migrate through the filters, thus indicating that the inhibition was not due to cell death This was also confirmed by the results of Trypan Blue exclusion tests.

It has previously been pointed out that leukocytes show enhanced non-directional locomotion when exposed to chemotactic agents in the absence of a concentration gradient (22 23). This type of stimulated locomotion has recently been termed chemokinesis (13) as distinct from chemotaxis which is the stimulated migration in the direction of a concentration gradient. The leukotactic response observed in a micropore filter system has been shown to be the result of the combined effects of chemotaxis and chemokinesis (21 22). Also the inhibition of this response may be due to either inhibition of chemotaxis, chemokinesis, or both.

The observation that demecolcine strongly inhibited the migration of PMN towards casein in Boyden chambers while affecting the velocity of PMN moving randomly on a glass slide to a much lesser extent (4) has supported the theory that intact microtubules are important for the chemotactic response of neutrophils (1) The experiments shown in Table 1 were designed to explore these results further (4) by analysing the separate effects of colchicine on PMN spontaneous motility chemotaxis and chemokinesis in a Boyden chamber using a checkerboard assay It was therefore surprising that colchicine had a strong inhibitory effect on neutrophil chemokinesis, while the cells retained their ability to discern a chemotactic gradient and to respond to this with directional locomotion However the results are consistent with the data, but not with their interpretation in this study (4). Furthermore, the observation that colchicine-treated and untreated neutrophils migrated equally well in the absence of chemotactic factors on either side of the filter is also consistent with previous findings (4).

In one study colchicine was found to inhibit chemotaxis but not chemokinesis of lymphoblasts in a similar checkerboard assay (20). The difference between this observation and the present results may arise from the fact that the study on lymphoblasts used a front-line technique (22) for measuring leukocyte locomotion, while in the present study the number of migrating cells was considered It is also possible, although less likely that lymphoblasts may differ from granulocytes in the way in which they are affected by colchicine, or that they use different mechanisms for sensing and reacting to chemotactic stimuli

It is believed that the topographical distribution of certain cell membrane units is actively regulated and that this regulation is controlled by microtubules. Thus Concanavalin-A (Con-A) receptors show an essentially uniform distribution on the normal PMN surface. However pre-incubation of PMN with colchicine or other antitubulins permits movement of the Con-A-receptor complexes in the cell membrane to form caps at one end of the cell (16 17) It has been shown (15) that pre-incubation of PMN with a urate crystal-induced serum chemotactic factor inhibits this Con-A capping in colchicine treated cells. It was proposed that the chemotactic factor might induce polymerization of tubulin into microtubules, thus preventing colchicine from binding to tubulin. Also exposure of PMN to a complement-derived chemotactic factor has been shown morphologically to increase the number of microtubules (12)

From these results it seemed possible that pre incubation of PMN with a chemotactic factor might prevent or diminish the inhibition by colchicine of their chemokinetic response However pre-incubation of PMN with BCF did not result in any change in their sensitivity to colchicine as compared to control cells not pre-incubated with BCF as shown in Fig 2. This observation might be because in contrast to certain other chemotactic factors, BCF may not induce microtubule assembly in the PMN This interpretation implies that the induction of microtubule assembly is not a necessary event in the locomotory response of the PMN. However from this observation it might also be possible to argue that the colchicine inhibition of PMN chemokinesis does not depend on its antitubulin activity

In conclusion this study has shown that colchicine inhibits the locomotory response of PMN at very low concentrations in agreement with previous observations However the findings suggest that the inhibition of colchicine affects chemokinesis rather than chemotaxis, leaving the spontaneous motility unaffected The results of the study are consistent with the hypothesis that the integrity of the microtubule function is not necessary for the ability of the PMN to respond chemotactically Thus the inhibitory effect of colchicine on chemokinesis may not depend on its antitubulin effect It is possible that colchicine may affect the still unidentified membrane transport mechanism involved in the translation of the recognition signal into an appropriate locomotory response

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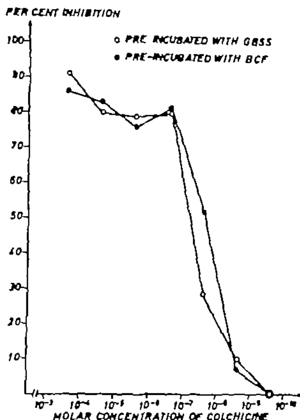


Fig. 2 Dose-dependent inhibition by colchicine of the attraction of PMN with BCF (diluted 1:3 with Gey's solution) The cells were pre-incubated either with BCF (diluted 1:3) (closed circles) or with Gey's solution (open circles) Abscissa. Molar concentration of colchicine. Ordinate. Percentage inhibition of activity Mean of two experiments.

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The observation that demecolcine strongly inhibited the migration of PMN towards casein in Boyden chambers while affecting the velocity of PMN moving randomly on a glass slide to a much lesser extent (4) has supported the theory that intact microtubules are important for the chemotactic response of neutrophils (1). The experiments shown in Table 1 were designed to explore these results further (4) by analysing the separate effects of colchicine on PMN spontaneous motility chemotaxis and chemokinesis in a Boyden chamber using a checkerboard assay It was therefore surprising that colchicine had a strong inhibitory effect on neutrophil chemokinesis while the cells retained their ability to discern a chemotactic gradient and to respond to this with directional locomotion. However the results are consistent with the data, but not with their interpretation in this study (4) Furthermore the observation that colchicine-treated and untreated neutrophils migrated equally well in the absence of chemotactic factors on either side of the filter is also consistent with previous findings (4).

In one study colchicine was found to inhibit chemotaxis but not chemokinesis of lymphoblasts in a similar checkerboard assay (20). The difference between this observation and the present results may arise from the fact that the study on lymphoblasts used a front line technique (22) for measuring leukocyte locomotion while in the present study the number of migrating cells was considered. It is also possible, although less likely that lymphoblasts may differ from granulocytes in the way in which they are affected by colchicine, or that they use different mechanisms for sensing and reacting to chemotactic stimuli.

It is believed that the topographical distribution of certain cell membrane units is actively regulated and that this regulation is controlled by microtubules. Thus Concanavalin-A (Con-A) receptors show an essentially uniform distribution on the normal PMN surface. However pre-incubation of PMN with colchicine or other antitubulins permits movement of the Con-A-receptor complexes in the cell membrane to form caps at one end of the cell (16 17). It has been shown (15) that pre-incubation of PMN with a urate crystal-induced serum chemotactic factor inhibits this Con-A capping in colchicine treated cells. It was proposed that the chemotactic factor might induce polymerization of tubulin into microtubules, thus preventing colchicine from binding to tubulin. Also exposure of PMN to a complement-derived chemotactic factor has been shown morphologically to increase the number of microtubules (12).

From these results it seemed possible that pre-incubation of PMN with a chemotactic factor might prevent or diminish the inhibition by colchicine of their chemokinetic response. However pre-incubation of PMN with BCF did not result in any change in their sensitivity to colchicine as compared to control cells not pre-incubated with BCF as shown in Fig 2. This observation might be because in contrast to certain other chemotactic factors, BCF may not induce microtubule assembly in the PMN. This interpretation implies that the induction of microtubule assembly is not a necessary event in the locomotory response of the PMN. However from this observation it might also be possible to argue that the colchicine inhibition of PMN chemokinesis does not depend on its antitubulin activity.

In conclusion this study has shown that colchicine inhibits the locomotory response of PMN at very low concentrations in agreement with previous observations. However the findings suggest that the inhibition of colchicine affects chemokinesis rather than chemotaxis, leaving the spontaneous motility unaffected. The results of the study are consistent with the hypothesis that the integrity of the microtubule function is not necessary for the ability of the PMN to respond chemotactically. Thus the inhibitory effect of colchicine on chemokinesis may not depend on its antitubulin effect. It is possible that colchicine may affect the still unidentified membrane transport mechanism involved in the translation of the recognition signal into an appropriate locomotory response.

This study was supported by the *Danish Medical Research Council*. The excellent technical assistance of Mrs. *Hanne Tansdorf* and Mrs. *Louise Klingenberg* is gratefully acknowledged. Professor *O Behnke* and Dr *Chr Koch* are thanked for their critical review of the manuscript.

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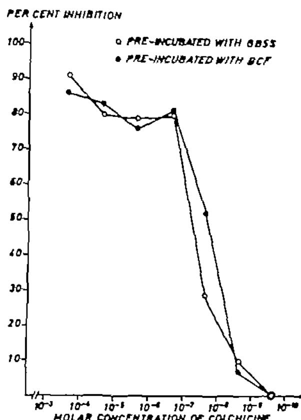


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Acta path. microbiol. scand. Sect. B, 86: 155–158, 1978

IN VITRO SENSITIVITY OF *TRICHOMONAS VAGINALIS* AND *CANDIDA ALBICANS* TO CHEMOTHERAPEUTIC AGENTS

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Strains of fresh clinical isolates of *Trichomonas vaginalis* and *Candida albicans* have been tested *in vitro* for their sensitivity to eight drugs used in the therapy of monilial and trichomonal vaginitis. Three of the chemotherapeutic agents, chlorchinaldol, clotrimazole and broxyquinoline, were effective against both organisms. Tinidazole and metronidazole were active against *T. vaginalis*. The strains of *C. albicans* were also sensitive to trichomycin, natamycin and nystatin. Tinidazole was the most effective trichomonacide, clotrimazole and chlorchinaldol were most effective against *C. albicans*, while chlorchinaldol had the best *in vitro* effect against both organisms.
The ranges of the MICs are compared to values previously reported.

Key words: Chemotherapeutic agents; *C. albicans*; *T. vaginalis*.

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The use of narrow spectrum chemotherapeutics is generally recommended to avoid many adverse effects, but in the treatment of gynecological disorders chemotherapeutics with a broad spectrum of activity have often the greatest therapeutic value. One of the reasons for this is that it is not always possible to perform an exact microbiological determination before starting therapy. In view of the association between monilial and trichomonal vaginitis, a therapy which promises to control both is desirable. Actually many of the drugs for these frequently occurring gynecological disorders are used for the treatment of both diseases (13). Thus a comparative test of their effectivity *in vitro* has been considered applicable. Such a test should use the same experimental conditions and take into account the fact that the response of the organisms may alter if not freshly isolated strains are used (3, 8, 14).

The present paper reports the results of sensitivity tests of seven respectively eight fresh clinical isolates of *T. vaginalis* and *C. albicans* to eight chemotherapeutic agents. The results are discussed in relation to effectiveness, therapeutic doses and minimum inhibitory concentrations. As isolates appear to vary in susceptibility, *in vitro* tests may have clinical utility.

MATERIALS AND METHODS

The freshly isolated strains of *T. vaginalis* and *C. albicans* were taken from a variety of vaginal specimens submitted to the Department of Medical Microbiology of the University of Turku.

Before use the *T. vaginalis* was grown on Trichomonas Medium CM 161 (Oxoid) containing sterile heat inactivated horse serum (80 ml/l), sodiumbenzylpenicillin (1000 units/ml) and streptomycin sulfate (500 µg/ml) at 37° C. The organism was seeded into 10 ml fresh medium and incubated for 48 hours. After shaking gently to obtain uniform distribution the number of viable organisms was estimated. The number of viable trichomonads was counted in a haematological counting chamber under a microscope at magnification of × 300. If the viability was good (at least 250 000 viable organisms per ml) 2 ml was added to 50 ml fresh

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TABLE 2. *Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of C. albicans as Determined According to Materials and Methods. Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

Strain of *C. albicans*	Clotrimazole (0.1–5)	Trichomycin (1–100)	Natamycin (1–50)	Chlorchinaldol (0.1–10)	Broxyquinoline (1–100)	Nystatin (1–100)	Conc. range tested (µg/ml)
	MIC (µg/ml)						
1	1	5	15	1	10	17	
2		1	5		15	13	
3	1	5	5	1	10	25	
4	1	1	5	5	10	10	
5	1	5	5	1	10	33	
6	1	5	5	1	10	13	
7	5	5	>50	1	10	13	
8	1	10	5	1	20	33	
Mean value	1.6	4.6	6.4	1.6	12	20	
Values given in literature (Ref.)	0.02–29.6 (2, 18, 7, 8, 9)	0.02–5 (19, 17, 3, 16)	3–6 (19, 3, 16)	1.5–6 (12)	4 (20)	1.5–10 (19, 3, 1, 2, 15, 18)	

DISCUSSION

The information provided by the pharmaceutical industry (13) about the clinical effectiveness of the drugs and the recommended therapeutic doses for the treatment of *T. vaginalis* and *C. albicans* infections is summarized in Table 3. All of the tested chemotherapeutic agents except nystatin are reported to be effective against a *T. vaginalis* infection. The *in vitro* response of the freshly isolated trichomonads to the drugs shows however that only five of them, metronidazole, clotrimazole, chlorchinaldol, broxyquinoline and tinidazole are trichomonacidal to the strains tested. Only one of the strains was sensitive to trichomycin while none was sensitive to natamycin. Numerous studies of the *in vitro* activity of the chemotherapeutic agents have been conducted by a number of investigators. It must be realized that the experimental conditions influence the results which has to be taken into consideration when data found in the literature are compared (3, 8, 14). For comparison reported MIC values for *T. vaginalis* have been included in Table 1. The values for metronidazole are in the range of our results although our MIC values are higher. *Paredes & Hawkins* (14) have also found natamycin

TABLE 3. *The sensitivity of* T. vaginalis *and* C. albicans *to the Tested Therapeutic Agents and the Recommended Therapeutic Doses According to Pharmaca Fennica (13)*

Chemotherapeutic agent		*T. vaginalis*	*C. albicans*	Recommended therapeutic dose (mg/24 hours) Systemic	Local
Metronidazole	(Flagyl®)	+		400–600	500
Clotrimazole	(Canesten®)	+	+		100
Trichomycin	(Trichomycin®)		+	150–300	150–300
Natamycin	(Pimafucin®)		+	300–400	25
Chlorchinaldol	(Sterosan®)	+	+		200
Broxyquinoline	(Starogyn®)	+	+		100
Tinidazole	(Tinigy ®)	+		300–500	
Polyvidone-iodine	(Betadine®)	+	+		200
Nystatin	(Nystacid®)		+	150	30

medium The viability was checked after *48 hours after* which the seeded media was transferred in 3 ml aliquots to small test tubes. Then 0 2 ml of a *serial dilution of a* drug was added. A standard tube without chemothera peutics was always included After 24 hours each tube was screened and the highest dilution of drug without any motile organisms was recorded as the minimal inhi bitory concentration (MIC) The standard tube contained 3–8 × 10^5 viable organisms per ml when the tubes containing a serial dilution of the drugs were screened. Each strain was tested for sensitivity to seven chemotherapeutics From the sample containing no motile trichomonads 0 2 ml was seeded to 3 ml fresh medium and the subculture was incubated for a further 48 hours for evaluation of the final results.

For determination of the minimum inhibitory concentration (MIC) for *C albicans* an agar dilution technique was used The diluted chemotherapeutics were mixed with melted 4% Saboraud's glucose agar (Merck) and poured into sterile petri plates. The yeast suspensions were prepared in sterile physiologic saline from 24 hours slope cultures grown at 37° C 0 1 ml of the yeast suspension containing about 1000–2000 cells per ml was transferred with a sterile pipette on the agar plate. Results were recorded after 48 hours at 37 C The lowest concentration of the chemotherapeutic that completely inhibited growth was taken as the MIC.

The drugs used were metronidazole (Flagyl®), clotrimazole (Canesten®), trichomycin (Trichomycin®) natamycin (Pimafucin®), chlorchinaldol (Sterosan®), broxyquinoline (Starogyn®), tinidazole (Tinigyn®) and nystatin (Nystacid®) Drug solutions were prepared in water or dimethylformamide (DMP) The final concentration of DMP had no effect on the growth of any of the organisms. One strain was always tested simultaneously against the different chemotherapeutic agents Nystatin was not tested for *T vaginalis* and metronidazole and tinidazole were not tested for *C. albicans*.

RESULTS

Table 1 shows the MIC values of the seven components tested for seven strains of *T vaginalis* All the strains were resistant to natamycin and only one of the seven strains was sensitive to trichomycin. The ranges of the MICs for the additional five chemotherapeutic agents, which have to be considered effective *in vitro* trichomonasides, were maximal for broxyquinoline and minimal for tinidazole. In general there was a great variability in the sensitivity of different strains of *T vaginalis* to a drug One of the strains was resistant to chlorchinaldol

The results of the examination of the antimycotic effects of the chemotherapeutic agents are shown in Table 2. The sensitivity of eight freshly isolated strains of *C albicans* was tested against six of the drugs. The ranges of the MICs were minimal with clotrimazole and chlorchinaldol and maximal with nystatin. One of the strains tested was resistant to natamycin. The strains of *C albicans* show less variation in their sensitivity to a chemotherapeutic agent as compared to the strains of *T vaginalis*.

TABLE 1 *Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of* T vaginalis *as Determined According to Materials and Methods Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

				MIC (µg/ml)				
Strain of *T vaginalis*	Metronidazole *(0 3–100)*	Clotrimazole *(5–666)*	Trichomycin *(10–666)*	Natamycin *(1–666)*	Chlorchinaldol (1–66)	Broxyquinoline (10–500)	Tinidazole *(0 1–10)*	Conc. range tested (µg/ml)
1	1	666	300	>666	50	400	2	
2	75	133	>666	>666	15	500	5	
3	15	333	>666	>666	50	400	0 3	
4	5	200	>666	>666	50	200	10	
5	5	200	>666	>666	50	100	2 5	
6	2	50	>666	>666	5	200	2	
7	5	50	>666	>666	>66	500	2 5	
Mean Value	8	260			37	330	3 5	
Values given in literature (Ref)	0 075–1 4 (11 14 4 5)	100 (18)	10 (9)	15–30 740 (16) (14)		40 (10)	0 5–1 0 (4 5)	

TABLE 2 *Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of C. albicans as Determined According to Materials and Methods. Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

	M.I.C. (µg/ml)						
Strain of *C. albicans*	Clotrimazole (0.1–5)	Trichomycin (1–100)	Natamycin (1–50)	Chlorchinaldol (0.1–10)	Broxyquinoline (1–100)	Nystatin (1–100)	Conc. range tested (µg/ml)
1	1	5	15	1	10	17	
2		1	5		15	13	
3	1	5	5	1	10	25	
4	1	1	5	5	10	10	
5	1	5	5	1	10	33	
6	1	5	5	1	10	13	
7	5	5	>50	1	10	13	
8	1	10	5	1	20	33	
Mean value	1.6	4.6	6.4	1.6	12	20	
Values given in literature (Ref.)	0.02–29.6 (2, 18, 7, 8, 9)	0.02–5 (19, 17, 3, 16)	3–6 (19, 3, 16)	1.5–6 (12)	4 (20)	1.5–10 (19, 3, 1, 2, 15, 18)	

DISCUSSION

The information provided by the pharmaceutical industry (13) about the clinical effectiveness of the drugs and the recommended therapeutic doses for the treatment of *T. vaginalis* and *C. albicans* infections is summarized in Table 3. All of the tested chemotherapeutic agents except nystatin are reported to be effective against a *T. vaginalis* infection. The *in vitro* response of the freshly isolated trichomonads to the drugs shows however that only five of them, metronidazole, clotrimazole, chlorchinaldol, broxyquinoline and tinidazole are trichomonacidal to the strains tested. Only one of the strains was sensitive to trichomycin while none was sensitive to natamycin. Numerous studies of the *in vitro* activity of the chemotherapeutic agents have been conducted by a number of investigators. It must be realized that the experimental conditions influence the results which has to be taken into consideration when data found in the literature are compared (3, 8, 14). For comparison reported MIC values for *T. vaginalis* have been included in Table 1. The values for metronidazole are in the range of our results although our MIC values are higher. *Paredes & Hawkins* (14) have also found natamycin

TABLE 3 *The sensitivity of* T. vaginalis *and* C. albicans *to the Tested Therapeutic Agents and the Recommended Therapeutic Doses According to Pharmaca Fennica (13)*

Chemotherapeutic agent		*T. vaginalis*	*C. albicans*	Recommended therapeutic dose (mg/24 hours) Systemic	Local
Metronidazole	(Flagyl®)	+		400–600	500
Clotrimazole	(Canesten®)	+	+		100
Trichomycin	(Trichomycin®)	+	+	150–300	150–300
Natamycin	(Pimafucin®)	+	+	300–400	25
Chlorchinaldol	(Sterosan®)	+	+		200
Broxyquinoline	(Starogyn®)	+	+		100
Tinidazole	(Tricangyn®)	+		300–500	
Polyvidone-iodine	(Betadine®)	+	+		200
Nystatin	(Mycostatin®)		+	150	30

medium. The viability was checked after 48 hours after which the seeded media was transferred in 3 ml aliquots to small test tubes. Then 0.2 ml of a serial dilution of a drug was added. A standard tube without chemotherapeutics was always included. After 24 hours each tube was screened and the highest dilution of drug without any motile organisms was recorded as the minimal inhibitory concentration (MIC). The standard tube contained 3–8 × 10^5 viable organisms per ml when the tubes containing a serial dilution of the drugs were screened. Each strain was tested for sensitivity to seven chemotherapeutics. From the sample containing no motile trichomonads 0.2 ml was seeded to 3 ml fresh medium and the subculture was incubated for a further 48 hours for evaluation of the final results.

For determination of the minimum inhibitory concentration (MIC) for *C. albicans* an agar dilution technique was used. The diluted chemotherapeutics were mixed with melted 4% Saboraud's glucose agar (Merck) and poured into sterile petri plates. The yeast suspensions were prepared in sterile physiologic saline from 24 hours slope cultures grown at 37° C. 0.1 ml of the yeast suspension containing about 1000–2000 cells per ml was transferred with a sterile pipette on the agar plate. Results were recorded after 48 hours at 37° C. The lowest concentration of the chemotherapeutic that completely inhibited growth was taken as the MIC.

The drugs used were metronidazole (Flagyl®), clotrimazole (Canesten®), trichomycin (Trichomycin®), natamycin (Pimafucin®), chlorchinaldol (Sterosan®), broxyquinoline (Starogyn®), tinidazole (Tinigyn®) and nystatin (Nystacid®). Drug solutions were prepared in water or dimethylformamide (DMP). The final concentration of DMP had no effect on the growth of any of the organisms. One strain was always tested simultaneously against the different chemotherapeutic agents. Nystatin was not tested for *T. vaginalis* and metronidazole and tinidazole were not tested for *C. albicans*.

RESULTS

Table 1 shows the MIC values of the seven components tested for seven strains of *T. vaginalis*. All the strains were resistant to natamycin and only one of the seven strains was sensitive to trichomycin. The ranges of the MICs for the additional five chemotherapeutic agents, which have to be considered effective *in vitro* trichomonasides, were maximal for broxyquinoline and minimal for tinidazole. In general there was a great variability in the sensitivity of different strains of *T. vaginalis* to a drug. One of the strains was resistant to chlorchinaldol.

The results of the examination of the antimycotic effects of the chemotherapeutic agents are shown in Table 2. The sensitivity of eight freshly isolated strains of *C. albicans* was tested against six of the drugs. The ranges of the MICs were minimal with clotrimazole and chlorchinaldol and maximal with nystatin. One of the strains tested was resistant to natamycin. The strains of *C. albicans* show less variation in their sensitivity to a chemotherapeutic agent as compared to the strains of *T. vaginalis*.

TABLE 1. Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of T. vaginalis *as Determined According to Materials and Methods. Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

	MIC (µg/ml)							
Strain of *T. vaginalis*	Metronidazole (0.3–100)	Clotrimazole (5–666)	Trichomycin (10–666)	Natamycin (1–666)	Chlorchinaldol (1–66)	Broxyquinoline (10–500)	Tinidazole (0.1–10)	Conc. range tested (µg/ml)
1	1	666	300	>666	50	400	2	
2	25	333	>666	>666	15	500	5	
3	15	333	>666	>666	50	400	0.3	
4	5	200	>666	>666	50	200	10	
5	5	200	>666	>666	50	100	2.5	
6	2	50	>666	>666	5	200	2	
7	5	50	>666	>666	>66	500	2.5	
Mean Value	8	260			37	330	3.5	
Values given in literature (Ref.)	0.075–1.4 (11, 14, 4, 5)	100 (18)	10 (9)	15–30, 740 (16) (14)		40 (10)	0.5–1.0 (4, 5)	

TABLE 2. *Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of C. albicans as Determined According to Materials and Methods. Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

Strain of *C. albicans*	Clotrimazole (0.1–5)	Trichomycin (1–100)	Natamycin (1–50)	Chlorchinaldol (0.1–10)	Broxyquinoline (1–100)	Nystatin (1–100)	Conc. range tested (µg/ml)
							M.I.C. (µg/ml)
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2		1	5		15	13	
3	1	5	5	1	10	25	
4	1	1	5	5	10	10	
5	1	5	5	1	10	33	
6	1	5	5	1	10	13	
7	5	5	>50	1	10	13	
8	1	10	5	1	20	33	
Mean value	1.6	4.6	6.4	1.6	12	20	
Values given in literature (Ref.)	0.02–29.6 (2, 18, 7, 8, 9)	0.02–5 (19, 17, 3, 16)	3–6 (19, 3, 16)	1.5–6 (12)	4 (20)	1.5–10 (19, 3, 1, 2, 15, 18)	

DISCUSSION

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Broxyquinoline	(Starogyn®)	+	+		100
Tinidazole	(Tinigyn®)	+		300–500	
Polyvidone-iodine	(Betadine®)	+	+		200
Nystatin	(Nystacid®)		+	150	30

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For determination of the minimum inhibitory concentration (MIC) for *C albicans* an agar dilution technique was used The diluted chemotherapeutics were mixed with melted 4% Saboraud's glucose agar (Merck) and poured into sterile petri plates. The yeast suspensions were prepared in sterile physiologic saline from 24 hours slope cultures grown at 37° C. 0 1 ml of the yeast suspension containing about 1000–2000 cells per ml was transferred with a sterile pipette on the agar plate. Results were recorded after 48 hours at 37° C The lowest concentration of the chemotherapeutic that completely inhibited growth was taken as the MIC.

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TABLE 1 *Minimum Inhibitory Concentration (MIC) of Different Chemotherapeutic Agents for Freshly Isolated Strains of T vaginalis as Determined According to Materials and Methods. Corresponding Values Reported in the Literature Are Given at the Bottom of the Table*

	M I C. (µg/ml)							
Strain of *T vaginalis*	Metronidazole (0.3–100)	Clotrimazole (5–666)	Trichomycin (10–666)	Natamycin (1–666)	Chlorchinaldol (1–66)	Broxyquinoline (10–500)	Tinidazole (0 1–10)	Conc range tested (µg/ml)
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2	25	333	>666	>666	15	500	5	
3	15	333	>666	>666	50	400	0 3	
4	5	200	>666	>666	50	200	10	
5	5	200	>666	>666	50	100	2.5	
6	2	50	>666	>666	5	200	2	
7	5	50	>666	>666	>66	500	2.5	
Mean Value	8	?60			37	330	3 5	
Values given in literature (Ref)	0 075–1 4 (11 14 4 5)	100 (18)	10 (9)	15–30 740 (16) (14)		40 (10)	0 5–1 0 (4 5)	

NITROBLUE TETRAZOLIUM (NBT) REDUCTION BY BACTERIA EMPLOYED FOR RAPID DETERMINATION OF ANTIBIOTIC CONCENTRATIONS IN SERUM

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Urban, T & Jarstrand, C Nitroblue tetrazolium (NBT) reduction by bacteria employed for rapid determination of antibiotic concentrations in serum Acta path microbiol scand Sect B, 86 159 164 1978

The ability of bacteria to reduce nitroblue tetrazolium (NBT) to formazan, and the inhibition of this process by antibiotics, have been utilized in a method to determine antibiotic concentrations in serum *Escherichia coli* growing in the presence of increasing but subinhibitory concentrations of antibiotics, produces diminished amounts of formazan This is photometrically quantified and the optical density obtained with the sample is compared with those obtained with known concentrations Levels of aminoglycosides below 1 μg/ml can be measured in the presence of other antibiotics also and the assay can be carried out in three hours Good correlation was obtained when 49 serum samples containing varying concentrations of sisomicin were tested simultaneously by the NBT assay and a disc diffusion method

Key words nitroblue tetrazolium, aminoglycosides, antibiotic concentration

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Because of the toxicity of aminoglycoside antibiotics, assay methods rapid enough for the results to be available in time to decide the size and time of the next dose are called for especially when an impaired kidney function may result in noxious concentrations even with standard doses Such methods have been envisaged (1 4 9), but are generally connected with technical difficulties which make them cumbersome for routine use in small laboratories

A photometric method for rapid assay of antibiotic levels in serum has now been developed by utilizing the ability of bacteria to reduce the almost colourless compound nitroblue tetrazolium (NBT) to dark blue formazan (3), and the ability of antibiotics to inhibit this reaction Unlike other metabolic activities employed for this purpose, such as urea (5) and glucose (8) splitting this process can be curtailed promptly without interfering with the quantification of the metabolite, thus allowing measurement to be performed when the reaction system is at a steady state.

MATERIALS AND METHODS

Procedure

Test organism A clinical isolate of *Escherichia coli* was used, with an MIC for gentamicin and sisomicin of 2 μg/ml It is fairly sensitive to cephalosporins (MIC for cephalotin 15 μg/ml) but resistant to therapeutically attainable levels for benzylpenicillin, ampicillin, carbenicillin cloxacillin, lincomycin and clindamycin, as determined by a disc diffusion method (2) The multiple resistance of the strain is probably mediated by R factor For this reason, the selective pressure must be maintai-

singularly ineffective as a trichomonacide *in vitro*

The reason for the consistently high MIC values has to be explained by a difference in the assay technique compared to previously reported tests. Usually a *T vaginalis* suspension has been added to a serial dilution of the trichomonacide to be tested and a microscopical examination has been performed after incubation for 2–3 days at 37° C to record the highest dilution without motile organisms (11 14 5). In our assay a serial dilution of the trichomonacide was added first when good viability and growth had been recorded and the tubes were screened for motile organisms after 24 h at 37° C. The latter method obviously requires a higher concentration of drug to kill all organisms.

Polyvidone iodine is also commonly used for treatment of both monilial and trichomonal vaginitis (13) but our assay methods are unsuitable for an *in vitro* comparison of this compound. The liberated inorganic iodine reacts with proteins in the medium and hence reduces the activity of polyvidone-iodine in the *in vitro* test. *Gershenfeld* (6) has reported on the effectivity of a vaginal gel containing 10% polyvidone iodine against *T vaginalis* and *C albicans*

Six of the tested chemotherapeutic agents are reported to be active against *C albicans* (13) This was confirmed in the *in vitro* test. Previously reported values for the susceptibility of *C albicans* to the tested agents (Table 2) are very much similar to our own. One *C albicans* strain of the eight that was tested showed resistance to natamycin and one required a much higher concentration that has been reported as the MIC (3–6 μg/ml). Although the material is very small this could be an indication of the development of resistance.

Of the five chemotherapeutics that are supposed to show clinical applicability against both organisms, only three, clotrimazole, chlorchinaldol and broxyquinoline appeared to be active in the *in vitro* test. The recommended therapeutic doses seems resonable (11 5) although the MIC values for *T vaginalis* are much higher than the corresponding values for *C albicans*

We are indebted to Dr *Simo Virtanen* for providing the freshly isolated strains of organisms, to the firms concerned for supplies of drugs and to Mrs. *Elsmarie Nyman* for help with the manuscript

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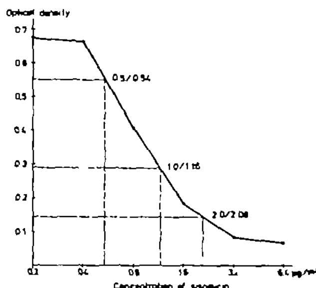


Fig 1 Dose-response curve for *E coli* growing in various concentrations of sisomicin. Expected/obtained values (µg/ml) for artificial samples are presented.

ned in subcultures This was done by placing paper discs, each containing one of the antibiotics, in a circle of 2 cm diameter on an agar plate. After overnight incubation the inoculum was taken from the growth in the centre of the circle. The strain was also found to be resistant to the bactericidal action of human serum, according to *Olling et al* (6)

Antibiotics. Gentamicin and sisomicin, both supplied as sulphates by Schering Corp Baltimore, USA, have been assayed

Standard series. Two-fold dilutions of the two drugs, from 6 4 to 0 2 µg/ml, were prepared in pooled heat inactivated human normal serum.

Test samples. Artificial samples with 0 5 1 0 and 2.0 µg/ml were made up in the same serum as above.

The assay system An overnight culture (Nutrient Broth No 2, Oxoid) of the test strain was centrifuged, and the bacteria were resuspended in an equal volume of the same medium, enriched with 2 per cent glucose and adjusted to pH 7 9 Such a suspension will contain about 2 × 10⁸ colony forming units (CFU) per ml, as determined by colony counts from serial dilutions. 0.5 ml of this suspension was dispensed into each of a suitable number of the test tubes. 0 5 ml of standard antibiotic solutions, or the same volume of the samples, was added to the tubes and the mixture was preincubated for 1½ h during agitation in a 37°C water bath Thereafter 1 ml of 0 1 per cent NBT (BDH Chemical Ltd, Poole, England) in Hank s solution was added to each tube and incubation was continued for 45 min, after which the reaction was curtailed by the addition of 1 ml 0.5 M HCl. The tubes were then centrifuged at 1000 g

Fig 2 Effect of enrichment of the medium and pH change on NBT reduction by *E coli* in the presence of various concentrations of sisomicin and gentamicin. Inoculum 2 × 10⁸ CFU/ml.

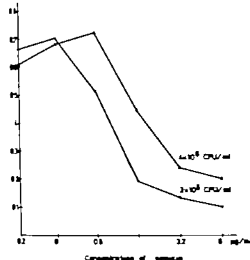


Fig 3 Effect of increased inoculum on NBT-reduction by *E. coli* in the presence of various concentrations of sisomicin

for 15 min, the supernates were discarded and the pellets eluted with 3 ml dimethylsulphoxide (DMSO) The optical densities (OD) of the extracts were determined at 572 nm by a filter photometer (Vitatron Manual DCP)

Calculation By comparing the OD obtained for the sample with the dose-response curve recorded with the standard series, the concentration of the antibiotic in the former was calculated graphically as shown in Fig 1

Experimental

The procedure described was adopted after preparatory experiments arranged in order to establish optimum conditions for the reaction

Medium Nutrient broth was used both plain and enriched with 2 per cent glucose, at its original pH of 6 9 or adjusted to pH 7 9 The ODs obtained with these media in the presence of different concentrations of gentamicin and sisomicin are given in Fig 2 Evidently the addition of glucose and the higher pH of 7 9 optimal for the action of aminoglycosides, favoured the NBT reduction as well as the inhibitory effect of the antibiotics and thus increased the OD range available for the measurements

Inoculum With the inoculum of 2 × 10⁸ *E. coli*/ml used in the standard procedure, 3 2 µg/ml of sisomicin diminished the NBT reduction to minimum, while 0 4 µg/ml permitted maximum activity When the inoculum was doubled, the NBT reduction was not significantly increased, but the levels of sisomicin needed for inhibition were also doubled as seen from Fig 3 leading to diminished sensitivity of the system In some experiments, the volume of bacterial suspension was increased at the expense of the serum volume The standard curve then became steeper but the sensitivity of the test system was again diminished.

Pre-incubation The differences between the amounts of formazan produced at the different concentrations of the antibiotic were evident already after pre-incubation for 1 h and were maximal after 1½ h Further prolongation resulted in only a slight increase of the range, as seen from Fig. 4.

Reproducibility of the method

Ten artificial sisomicin serum samples containing 0 5 1 0 1.5 2 0 2.5 3.0 3 5 4 0 4.5 and 5 0 µg/ml were prepared. Samples below 2 0 µg/ml were analysed only undiluted, samples between 2.0 and 3 0 µg/ml undiluted and in dilution 1 2, and samples with more than 3 0 µg/ml undiluted and in dilutions 1.2 and 1 4 0.5 ml aliquots of each sample and its dilutions were distributed into ten tubes, frozen to −18°C and analysed according to the procedure described previously on 10 different days. Standard series were prepared and kept in the same manner

Influence of other antibiotics. To samples with 1 0 and 2.0 µg/ml of gentamicin were added other antibiotics in four or five different concentrations Benzyl penicillin 25 − 400 IU/ml, ampicillin 3 125 − 100 µg/ml, carbenicillin 15 6 − 500 µg/ml, cloxacillin 2.5 − 40 µg/ml, lincomycin 2 5 − 20 µg/ml and clindamycin 1 25 − 10 µg/ml The artifical samples containing two antibiotics were assayed according to the procedure described previously

Comparison by Disc Diffusion Assay

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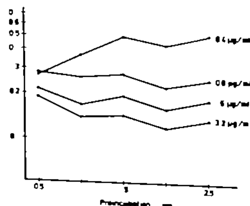


Fig 4 Effect of preincubation time on NBT-reduction by *E. coli* growing in the presence of various concentrations of sisomicin.

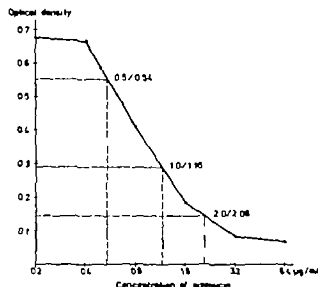


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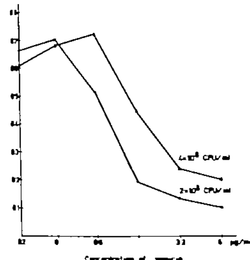


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for 15 min, the supernates were discarded and the pellets eluted with 3 ml dimethylsulphoxide (DMSO) The optical densities (OD) of the extracts were determined at 572 nm by a filter photometer (Vitatron Manual DCP)

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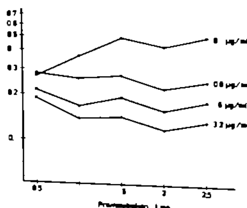


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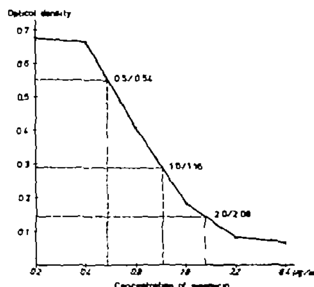


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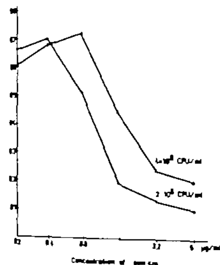


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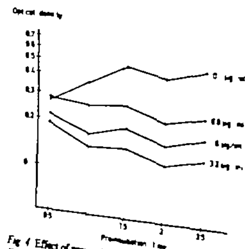


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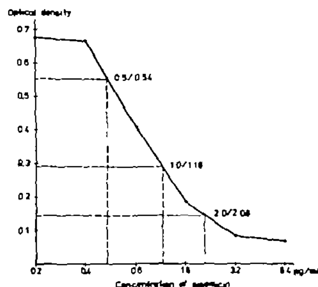


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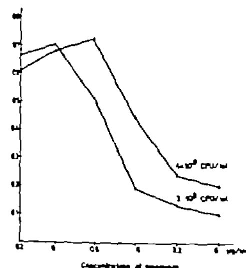


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Reproducibility of the method

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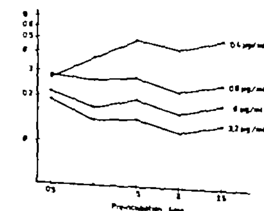


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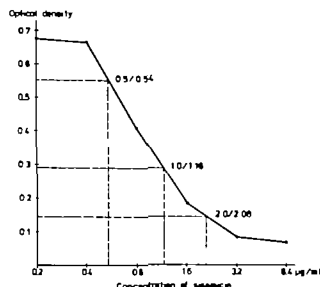


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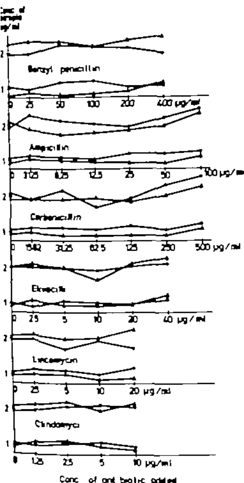
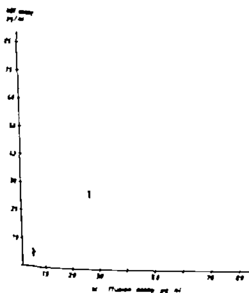


Fig 5 Assay values for artificial sisomicin (circles) and gentamicin (triangles) samples obtained in the presence of other antibiotics



DISCUSSION

Different bacterial species and strains have different degrees of NBT activity (3). The *E. coli* strain chosen as test organism has a high NBT reducing capacity and provides a dose-response curve steep enough to permit a high accuracy of the method. It is also sufficiently sensitive to the drugs to be assayed to permit even low concentrations to be determined, viz. down to 0 4 µg/ml. On the other hand, it is resistant to other antibiotics likely to be present together with aminoglycosides. Cephalosporins, to which the test strain is susceptible, are not likely to be used together with aminoglycosides in our hospital. If such combinations are to be used, a cephalosporin resistant test strain must be employed. However as in all biological assay methods, other cytostatic drugs than antibiotics may be suspected of interfering with the reaction.

Samples of unpredictable concentrations must be assayed in two or three dilutions to make sure that some of them will fall within the measurable range of the method, but a serum volume of 1 ml will always be sufficient. As the test strain is resistant to the bactericidal action of human serum in the concentration (50 per cent) existing in the test and for the 2 h of incubation, no interference of serum factors is likely to take place.

When assays were performed by the NBT method and the disc diffusion test, both of which measure biologically active drugs, good correlation was obtained. The method has been used in connection with clinical evaluations of sisomicin and netilmicin. As compared to other assay methods, such as agar well, disc or cup diffusion tests, methods based on enzyme activities such as urea or glucose splitting, assays of split products from enzymatic decomposition or radio-immunosorbent assays, the method now described has some advantages. It is rapid and simple and does not require expensive equipment or specially trained personnel. Thus it is suitable for use in small laboratories.

Fig 6 Comparison between the NBT and disc diffusion assays for measurement of sisomicin in serum
r = 0 96
slope = 0 78
y intercept = 0 43

TABLE 1 *Assay Values for Artificial Sisomicin and Gentamicin Samples*

Antibiotic	Concentration prepared µg/ml	Optical density I	II	mean value	Concentration calculated µg/ml
Sisomicin	2.0	0 148	0.148	0 146	2 08
	1 0	0 288	0 286	0.287	1 16
	0 5	0 551	0.552	0 552	0.54
Gentamicin	2 0	0 162	0.158	0 160	1 92
	1 0	0 386	0 374	0 380	0 92
	0 5	0 539	0.550	0 545	0 48

RESULTS

In order to illustrate the performance of the test and the calculation artificial serum samples with three different concentrations of sisomicin or gentamicin were prepared and assayed. The ODs obtained for the standard series and for the samples are presented in Fig 1 also in Table 1 where the results of the determinations are given.

In order to test the reproducibility of the method one portion of each of the ten sisomicin samples containing ten different concentrations of the drug was analysed on 10 different days, with the results given in Table 2. The percentage standard deviations are about the same for all levels, with a mean value of 5 57 per cent, which expresses the reproducibility of the method.

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TABLE 2 *Reproducibility of the Method Results of Sisomicin Assays Repeated on 10 Different Days on Artificial Serum Samples*

Expected value µg/ml	Mean value of 10 experiments	Standard deviation	Standard deviation as percentage of the mean value
0 5	0 47	0 06	12 00
1 0	1 04	0 08	8 00
1 5	1.50	0 05	3 33
2 0	2.04	0 13	6.50
2 5	2 45	0 11	4 47
3 0	3 07	0 12	3 90
3 5	3 38	0 11	3 25
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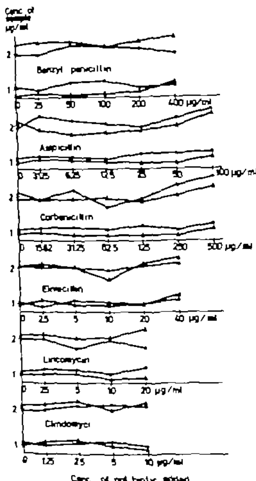


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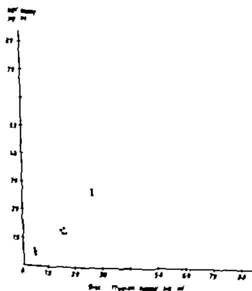


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TABLE 2 *Reproducibility of the Method Results of Sisomicin Assays Repeated on 10 Different Days on Artificial Serum Samples*

Expected value µg/ml	Mean value of 10 experiments	Standard deviation	Standard deviation as percentage of the mean value
0 5	0 47	0 06	12 00
1 0	1 04	0 08	8 00
1 5	1 50	0 05	3 33
2 0	2 04	0 13	6.50
2 5	2.45	0 11	4 47
3 0	3 07	0 12	3 90
3 5	3 38	0 11	3 25
4 0	4 09	0 30	7.32
4 5	4 92	0 18	3 65
5 0	5 13	0 27	5.25
		Mean value for the percentage standard deviations.	5 57

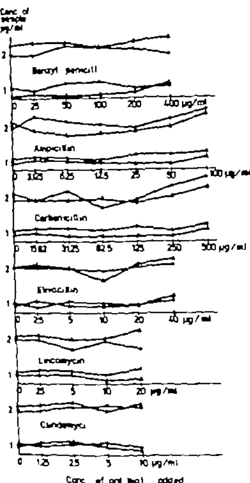


Fig. 5 Assay values for artificial sisomicin (circles) and gentamicin (triangles) samples obtained in the presence of other antibiotics

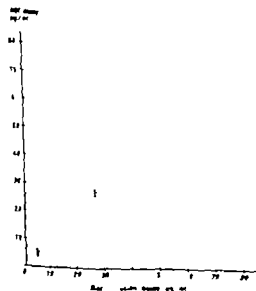


Fig. 6 Comparison between the NBT and disc diffusion assays for measurement of sisomicin in serum
r = 0.96
slope = 0.78
y intercept = 0.43

DISCUSSION

Different bacterial species and strains have different degrees of NBT activity (3). The *E. coli* strain chosen as test organism has a high NBT reducing capacity and provides a dose-response curve steep enough to permit a high accuracy of the method. It is also sufficiently sensitive to the drugs to be assayed to permit even low concentrations to be determined, viz. down to 0.4 µg/ml. On the other hand, it is resistant to other antibiotics likely to be present together with aminoglycosides. Cephalosporins, to which the test strain is susceptible, are not likely to be used together with aminoglycosides in our hospital. If such combinations are to be used, a cephalosporin resistant test strain must be employed. However, as in all biological assay methods, other cytostatic drugs than antibiotics may be suspected of interfering with the reaction.

Samples of unpredictable concentrations must be assayed in two or three dilutions to make sure that some of them will fall within the measurable range of the method, but a serum volume of 1 ml will always be sufficient. As the test strain is resistant to the bactericidal action of human serum in the concentration (50 per cent) existing in the test and for the 2 h of incubation, no interference of serum factors is likely to take place.

When assays were performed by the NBT method and the disc diffusion test, both of which measure biologically active drugs, good correlation was obtained. The method has been used in connection with clinical evaluations of sisomicin and netilmicin. As compared to other assay methods, such as agar well, disc or cup diffusion tests, methods based on enzyme activities such as urea or glucose splitting, assays of split products from enzymatic decomposition or radio-immunosorbent assays, the method now described has some advantages. It is rapid and simple and does not require expensive equipment or specially trained personnel. Thus it is suitable for use in small laboratories.

TABLE 1 *Assay Values for Artificial Sisomicin and Gentamicin Samples*

Antibiotic	Concentration prepared µg/ml	Optical density I	II	mean value	Concentration calculated µg/ml
Sisomicin	2 0	0.148	0 148	0 146	2 08
	1 0	0.288	0.286	0 287	1 16
	0 5	0.551	0.552	0 552	0.54
Gentamicin	2 0	0 162	0 158	0 160	1 92
	1 0	0 386	0.374	0 380	0 92
	0 5	0.539	0 550	0 545	0 48

RESULTS

In order to illustrate the performance of the test and the calculation, artificial serum samples with three different concentrations of sisomicin or gentamicin were prepared and assayed. The ODs obtained for the standard series and for the samples are presented in Fig 1 also in Table 1 where the results of the determinations are given.

In order to test the reproducibility of the method, one portion of each of the ten sisomicin samples containing ten different concentrations of the drug was analysed on 10 different days, with the results given in Table 2 The percentage standard deviations are about the same for all levels, with a mean value of 5 57 per cent, which expresses the reproducibility of the method

The influence of other antibiotics present in the sample together with 1 and 2 µg/ml of sisomicin or gentamicin was tested in the experiments reported in Fig. 5 The six antibiotics were used in four or five different concentrations. When a sign test was performed for each of the six antibiotics, the values obtained with the additional antibiotic did not differ significantly from those obtained with only gentamicin or sisomicin, and there was no correlation between the concentration of the added antibiotic and the value obtained for the gentamicin or sisomicin concentration.

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3 0	3 07	0 12	3 90
3 5	3 38	0 11	3 25
4 0	4 09	0.30	7 32
4 5	4 97	0 18	3 65
5 0	5 13	0 27	5.25
	Mean value for the percentage standard deviations:		5.57

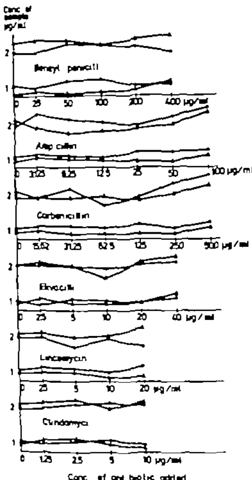


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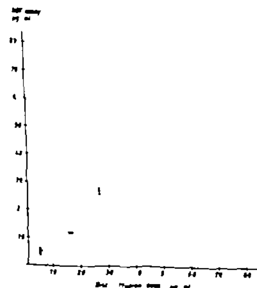


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Acta path. microbiol. scand. Sect. B, 86: 165–167, 1978

BRIEF REPORT

OBSERVATIONS ON THE ULTRASTRUCTURE OF THE SPOROCYST AND THE INITIATION OF SPOROZOITE FORMATION IN *TOXOPLASMA GONDII*

D. J. P. Ferguson[1,2], A. Birch-Andersen[2], J. Chr. Siim[1] and W. M. Hutchison[3]*

FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2], Statens Seruminstitut, Copenhagen, Denmark, and Department of Biology[3], University of Strathclyde, Glasgow, Scotland

Ferguson, D. J. P., Birch-Andersen, A., Siim, J. Chr. & Hutchison, W. M. Observations on the ultrastructure of the sporocyst and the initiation of sporozoite formation in *Toxoplasma gondii*. Acta path. microbiol. scand. Sect. B, 86: 165–167, 1978.

The ultrastructure of the immature sporocyst and the initiation of sporozoite formation in *T. gondii* was examined in oocysts which had been allowed to sporulate for 12 or 24 hours at 27°C. The sporocyst was ellipsoidal in shape and possessed a two layered sporocyst wall. A nucleus, with associated Golgi bodies, was situated at either end of the organism. The cytoplasm of the sporocyst also contained a number of polysaccharide granules, lipid globules, mitochondria and a few strands of rough endoplasmic reticulum. Sporozoite formation was initiated by the appearance of two dense plaques at either end of the organism in the vicinity of a nucleus, adjacent to the limiting membrane of the organism.

Key words: *Toxoplasma gondii*; initiation of sporozoite formation; sporocyst; ultrastructure.

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Received 8.ii.78. Accepted 17.iv.78.

It is known that *Toxoplasma gondii* is a coccidian parasite of the domestic cat (6). In this host the parasite gives rise to an *Isospora*-like oocyst which contains, after sporulation, two sporocysts each of which possesses four sporozoites. Until recently, the ultrastructural changes occurring during the sporulation of the oocysts of the family Eimeriidae have not been examined because of the resistance of the oocyst wall to the penetration of the fixing and embedding agents. However, a special preparative technique is now available, which allows the study of coccidian oocyst sporulation at the subcellular level (1). To date, the only coccidian studied using this technique has been a member of the genus *Eimeria*, *E. brunetti* (2, 3 & 4). To the best of our knowledge, no *Isospora*-like oocysts have been examined by this technique, but in this note the ultrastructure of the sporocyst and the initiation of sporozoite formation in *T. gondii* will be described.

Materials and Methods

Oocysts were obtained from the faeces of specific pathogen free (SPF) cats which had been infected with the SSI/119 strain of *T. gondii*. The oocysts were concentrated and allowed to sporulate at 27°C for either 12 or 24 hours before processing for electron microscopy. For this purpose the technique used was similar to that described previously for the oocysts of *E. brunetti* (1).

Results

In the material which has sporulated for 12 or 24 hours it is possible to find oocysts which had reached the early sporocyst stage of development. At this stage the

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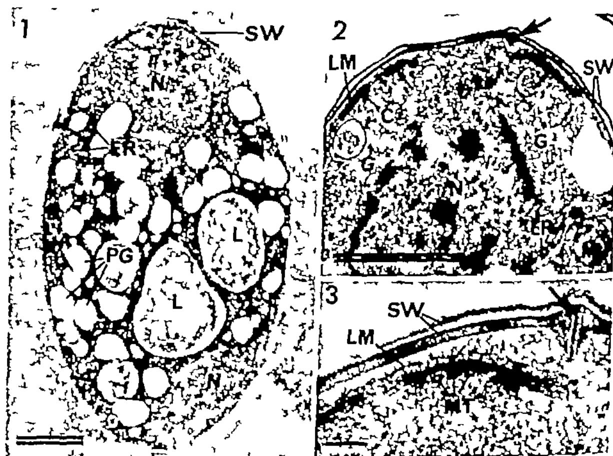
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cytoplasmic mass is ellipsoidal in shape and is enclosed by a unit membrane; external to this is the sporocyst wall. The sporocyst wall comprises a thin outer layer and an inner layer 40–45 nm thick consisting of four curved plates (Figs 2 & 3). The cytoplasmic mass possesses two nuclei situated at opposite ends of the organism. Golgi bodies are found adjacent to the nuclei and the cytoplasm also contains a number of polysaccharide granules, lipid globules, mitochondria and a few strands of rough endoplasmic reticulum (Figs 1 & 2).

The first indication of *sporozoite formation* is the appearance of two dense plaques at either end of the organism. These plaques are always observed in the region of a nucleus and adjacent to the limiting membrane of the organism (Fig. 2). A conoid is present in the central part of the plaques (Fig. 2). At higher magnification the plaques are found to consist of two closely applied unit membranes with underlying microtubules (Fig. 3) and they thus represent the anlage of the inner complex of the pellicle of each of the sporozoites.

Discussion

The structure of the two layered sporocyst wall of *T. gondii* with the inner layer consisting of four separate plates is similar to that reported for *Isospora canis* (1) and *I. endocallimici* (9). The appearance and distribution of the intracytoplasmic organelles of the early sporocyst



Figures 1–3 are micrographs of early sporocysts of *T. gondii* observed within oocysts which had sporulated for either 12 or 24 hours at 27°C. A single bar (–) on a micrograph represents 100 nm and a double bar (=) represents 1 µm.

Fig. 1. A longitudinal section through an early sporocyst in which the sporocyst wall (SW) is visible. A nucleus (N) is present at either end of the organism. In addition, the cytoplasm contains a number of polysaccharide granules (PG), lipid globules (L) and some rough endoplasmic reticulum (ER). × 15 000

Fig. 2. A section through one end of a sporocyst showing the two dense plaques (small arrows) close to the limiting membrane (LM) in the region of a nucleus (N). Note the presence of a conoid (C) in the centre of one plaque. The sporocyst wall (SW) and a junction between two of the plates constituting its inner layer can be seen (large arrow). Golgi bodies (G), a mitochondrion (MI) and some rough endoplasmic reticulum (ER) are also visible. × 30 000

Fig. 3. A higher magnification through the region of a plaque. The plaque can be seen to consist of two closely applied unit membranes (small arrows) with underlying microtubules (MT) situated adjacent to the limiting membrane (LM). The two layered sporocyst wall (SW) is visible and a junction region between two plates of the inner layer is also illustrated (large arrow). × 90 000

of *T gondii* is similar to that reported for the early sporocyst of *Sarcocystis tenella* (7), although a somewhat better cytoplasmic preservation is obtained using the present technique. The sporocyst of *T gondii*, however lacks both the crystalloid body observed in *S tenella* (7) and the dense bodies observed in *E. brunetti* (2 & 4).

The first evidence of sporozoite formation within the sporocysts of *T gondii* was the appearance of two dense plaques with associated conoid and microtubules at each end of the cell. These plaques lie close to the limiting membrane in the vicinity of each nucleus. Thus the initiation of sporozoite formation is similar to that reported for *E brunetti* (2 & 4), but in *E brunetti* only one plaque is associated with each nucleus. The endogenous forms of *T gondii* multiply by either endodyogeny or endopolygeny (5). In both processes daughter formation is initiated in the interior of the mother cell and is occurring during and associated with the final nuclear division. Thus each nucleus is divided between two internally developing daughters. It would appear from the observations available, that during sporozoite formation of *T gondii* the sporozoites will be formed in association with the final nuclear division. However this process differs from that reported for the endogenous forms in that sporozoite formation is initiated close to the limiting membrane of the sporocyst.

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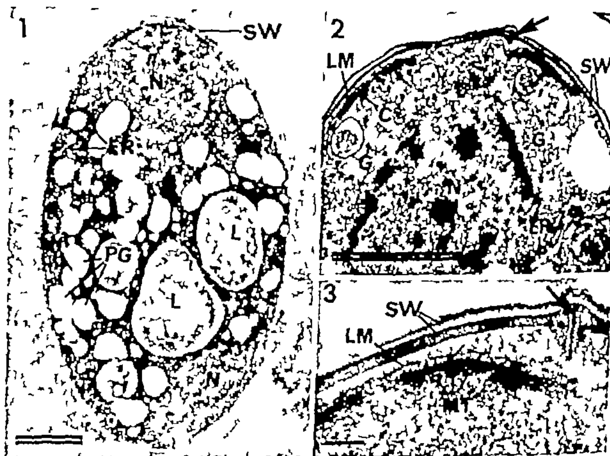
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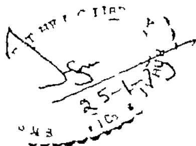
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Section B MICROBIOLOGY



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Section B Microbiology

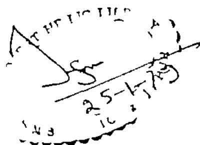
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ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
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Brief Report.

ANTIGENIC CHARACTERISTICS OF *MORAXELLA NONLIQUEFACIENS* FIMBRIAE IN DOUBLE IMMUNODIFFUSION

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Key words: Fimbriae; pili; double immunodiffusion; protein antigen denaturation; *Moraxella*; *Neisseriaceae*.

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The present report describes mainly the antigenic properties of fimbriae from a strain of *M. nonliquefaciens* studied by double immunodiffusion (Ouchterlony).

MATERIALS AND METHODS

Microorganisms, antigen labelling and cultivation. Purified fimbrial antigen used for immunization was prepared exclusively from *M. nonliquefaciens* NCTC 7784 SC-c, which is richly fimbriated (14) and highly competent in genetic transformation (3). SC or fim indicate fimbriated variants, whereas N indicates a low- or non-fimbriated one. The following strains were used as sources of whole-cell antigens in immunodiffusion experiments: *M. nonliquefaciens* 7784 SC-c, 3067/66 N-a and SC-a (4, 6), and 4663/62 SC-a (5, 6); *M. bovis* 4 SC-a (5, 6), and ATCC 10900 fim; *M. atlantae* A1922, fim, fimbriated variant (8), here designated A1922 fim; *Kingella kingae* (22, 23) 4177/66 SC-a (6); and *Neisseria elongata* subsp. glycolytica 6171/75 SC-a (7). As explained below, several subcellular antigens of strain 7784 SC-c (crude antigens, CA, CAcc and CAcy; purified antigens PA1, PA2, PA2cc and PA2cy) were used and often appear without strain designation. Antigens were also prepared from strain 3067/66 N-a

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ANTIGENIC CHARACTERISTICS OF *MORAXELLA NONLIQUEFACIENS* FIMBRIAE IN DOUBLE IMMUNODIFFUSION

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Electron microscopy. Negative staining was carried out with 0.8 per cent (w/v) sodium silicotungstate (SST), pH 7, as described previously (5, 14). KBr treated antigens and controls were diluted 100-fold with distilled water and centrifuged at 192,000 × g for 60 min in the Spinco SW 50.1 rotor. The sediments were suspended in the remnants of liquid after careful removal of supernatant. This suspension was added to a grid, left for one min, and removed before staining briefly.

Chemicals. The chemicals used were of analytical grade from various sources. The human serum pool was from Behringwerke AG, and the human plasma pool was provided by this institute.

RESULTS

Preparation and morphology of fimbrial antigens. The amount of cellular protein obtained from *M. nonliquefaciens* 7784 SC-c (50 blood agar plates) was about 90 mg. The average yields in terms of protein were 5.1 per cent for crude antigen (CA, CAcx), 2.7 per cent for purified antigen 2 (PA2, PA2cx) and 1.7 per cent for PA1. CAcy and PA2cy yields were lower. By SDS polyacrylamide gel electrophoresis, PA1 and PA2 showed mainly one protein band with an apparent molecular weight of 17 000. Some PA2cx and PA2cy preparations were virtually pure by this method and allowed amino acid sequence determination for 49 steps from the N-terminal end (16).

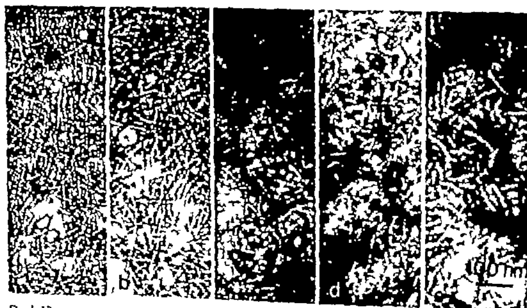


Fig. 1. Ultrastructural morphology of fimbrial antigens by negative staining. Purified antigen 1 (PA1) in Fig. 1a, purified antigen 2 (PA2) in Fig. 1b, and crude antigen (CA) in Fig. 1c. Non-fimbrial structures are marked with arrows. PA1 and PA2 were supplemented by 1 and 2 per cent formaldehyde (w/v), respectively, one day before sampling for electron microscopy. A fresh PA2cx antigen was mixed with KBr and divided into aliquots, one was treated by freeze-thawing 10 times (Fig. 1d) and the other was heated at 75°C for 20 min (Fig. 1e). The latter preparations were diluted and ultracentrifuged before negative staining. Arrowheads in Fig. 1e indicate a single fimbria. 100 000 ×

and SC-a, CAcx and PA2cx and the strain/variant is always indicated.

The bacteria were grown either on blood agar plates (5) or on Yeast Extract enriched Mueller Hinton agar plates, referred to as Mueller Hinton plates (14).

Fimbrial antigen preparations. A fimbrial suspension was prepared by harvesting the bacteria, homogenizing them and removing whole cells by differential centrifugation. The *combined supernatant* fraction described previously (14) was used either for the preparation of purified antigens (see below) or was centrifuged at 80 000 × g for 60 min (Spinco rotor 40) in the cold. *Crude fimbrial antigen, CA,* was obtained by suspending the pellet to 1/10 of the volume used for harvesting, in 0 01 M tris HCl, pH 8 0 containing 0 01 M NaN_3 (TA buffer pH 8). The preparation was used immediately or frozen at −25°C until use. A *control antigen* (CAcx) from bacteria grown on Mueller Hinton plates was prepared in a similar manner to exclude the possibility of blood components contaminating the fimbriae. A *second control antigen* (CAcy), also from Mueller Hinton grown bacteria, was further purified from media components by sedimenting the bacteria at 2 000 × g and washing them once. These supernatants were discarded. Homogenization and subsequent fractionation of the homogenates were then carried out as for CA and CAcx. *Purified fimbrial antigens* (PA1 and PA2). To obtain PA1 the combined supernatants (see above) were first rehomogenized and then centrifuged at 2 000 × g for 30 min. Fimbriae were removed from this last supernatant by precipitation with half a volume of cold methanol prechilled to −70°C as described by *Goodheart et al* (18). After centrifugation at 12 000 × g for 15 min the precipitated fimbriae were extracted by homogenization in TA buffer pH 7 5 at one half the volume used for harvesting and recentrifuged. The sediment was washed once by homogenization in 1/20 volume TA buffer pH 7 5 before dialysis and $MgCl_2$ precipitation of the extract by the method of *Buchanan et al* (11). A similar antigen, PA2, was made by directly precipitating the combined supernatants with methanol (see above) and dialyzing the TA buffer pH 7 5 extract, first against distilled water for 4 h and then against 0 1 M $MgCl_2$ containing 0 01 M NaN_3 for 16 h. Both PA1 and PA2 were then pelleted by centrifugation at 80 000 × g for 60 min, suspended in 1/25 volume TA buffer pH 8 and used immediately or kept frozen at −25°C *Control antigens* PA2cx and PA2cy were made from extracts of cells grown on Mueller Hinton plates as described for the crude antigens CAcx and CAcy followed by the procedure for PA2

Whole-cell antigens Bacteria grown for 16 to 24 h (exceptionally 48 h) on blood agar plates or on Mueller Hinton plates were harvested with a platinum loop and suspended in TA buffer pH 7 5 at a cell-protein concentration of approximately 40 mg/ml. The bacterial suspension was mixed by swirling on a Vortex Jr mixer and kept frozen These antigens were occasionally used in this form, but were usually treated as described below

Agar plate antigens. Dry samples of Mueller Hinton Broth (Difco), Yeast Extract (Difco), and Tryptose Blood Agar Base (Oxoid) were extracted individually with phosphate buffered saline (PBS) (14), 50 ml/3 g, at room temperature for 60 min and centrifuged or glass-wool-filtered. The soluble material was dialyzed against distilled water before lyophilization. For immunodiffusion, the residue was suspended in 0 8 ml PBS. Blood agar medium (complete) was homogenized in 30 ml PBS per plate and centrifuged, and the supernatant was dialyzed against water before lyophilization. The residue was dissolved in 1/20 of the original volume of PBS, giving the blood agar extract.

Treatment of antigens. When not used untreated small amounts of the fimbrial and whole-cell antigen preparations in corked 6 × 50 mm glass tubes were subjected to physical and chemical denaturating agents. An equal volume of TA buffer pH 7.5 or one of the different chemicals (see below) was added. Physical conditions used were freeze-thawing (dry ice/acetone and 20°C water) and temperatures of 0°C, and 75°C for 20 min, and 100°C for 120 min. Sonication was performed with a 1 ml volume in a 5 ml plastic tube for 30 s under ice cooling, and with a microtip probe (Model B-12 Sonifier 20 KHz, at 10 W output energy Branson, Danbury Conn.). The following chemicals were tested. 1 M KBr in TA buffer pH 7.5 3 M NaSCN in 0 01 M sodium phosphate buffer pH 6.5; and 8 M urea in distilled water freshly prepared.

Preparation of antisera PA1 from *M nonliquefaciens* 7784 SC-c at 1 mg protein/ml was treated with one per cent formaldehyde overnight at 4°C before it was mixed thoroughly with an equal volume of Incomplete Freund's Adjuvant (Difco) and injected intracutaneously into three rabbits (Nos. 1 3 5) The rabbits received five injections (25μg (protein) formalinized antigen/kg body weight) at two-week intervals. Thereafter the interval was increased to six weeks (19) with a total of eight injections Serum C was collected eight days after the fifth injection. D eight days after the sixth, E eight days after the seventh, F nine days after and H five weeks after the eighth injection. The number before the serum designation in the following indicates the number of the rabbit immunized. To remove material precipitating non-specifically near the antiserum well, the rabbit sera were pretreated with Aerosil 380 (Degussa) (33)

Protein determination. The method of *Schaffner & Weissmann* was used for protein determination (31) The reference protein was bovine serum albumin (fraction V Armour Pharmaceutical)

Double immunodiffusion. One per cent A-45 agarose (L'Industrie Biologique Française A A R.L) in PBS containing 0 0001 M merthiolate, 10 ml per 9 cm Petri dish, was used. The wells were usually 2 5 mm in diameter but 4 mm wells were also used occasionally The plates were incubated for from one to four days at 33°C in a moisture chamber though most precipitates were visible within four to six h Unstained plates were photographed by dark field illumination The plates were afterwards rinsed thoroughly with PBS overnight, treated with 0 2 per cent (w/v) tannic acid in distilled water for about 10 min (32) to intensify weak precipitates, and then studied again.

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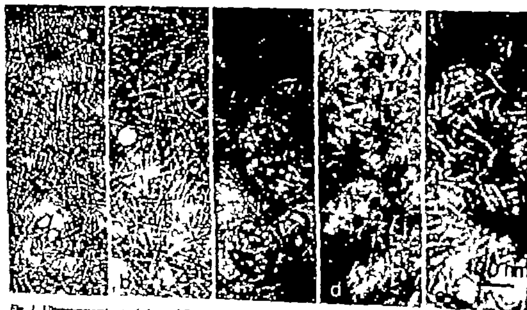


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Electron microscopy of formalin-treated PA1 antigen which (in several lots) was the only antigen used for immunization, shows a meshwork of fimbrial threads lying straight or slightly curved (Fig 1a) The diameter of the fimbriae ranges from 6 to 8 nm when filaments running in parallel are measured (4) The width of the light zone is about 4 nm Very few ends can be seen, but a few rounded structures of various sizes, presumably derived from the outer membrane (cf ref 15) are evident (arrows) The PA2 preparation shown in Fig. 1b seems to resemble PA1 in all respects. The crude antigen (CA) definitely contains more of the round structures (Fig 1c) and is mixed with some whole cells. This contamination may be reflected in the yields (see above).

PA2cx was also prepared from *M nonliquefaciens* 3067/66 SC-a and gave 0 53 per cent of rather impure fimbriae as compared to similar 7784 SC-c preparations. When the same method was applied to the N-a variant of 3067/66 the yield was 0 32 per cent, and subcellular material with no fimbriae was seen by electron microscopy This latter antigen was used as a fimbria free control antigen in some tests.

Double immunodiffusion with M *nonliquefaciens 7784 SC-c antigen.* The antibody response to formalinized PA1 was studied by immunodiffusion. The preimmune sera showed no activity to purified fimbriae, but after immunization three precipitates were detected (Fig. 2a and b top and lower right). With all rabbit antisera, both PA1 and PA2 gave rise to two main precipitate lines, I and II and the 1H antiserum showed a third less distinct line, III, marked with an arrow on the antiserum side of the precipitate labelled II Immunoprecipitates were also demonstrated with CA preparations, and there seemed to be fusion of CA lines with PA1 and PA2 lines as seen on Fig 2a, b and c. Faint precipitates were found with the untreated whole-cell extract of *M nonliquefaciens* 7784 SC-c, the source of the fimbrial antigen used for immunization. Also antigen prepared from cells grown on Mueller Hinton plates and washed (PA2cy) gave a similar response as PA2. The antisera showed no activity with the blood agar extract (BAE), yeast extract (YE) Mueller Hinton broth extract (MH), tryptose blood agar base (TBA) human serum (HS), human plasma (HP) or the untreated whole-cell antigen from a distantly related, fimbriated bacterium (6171

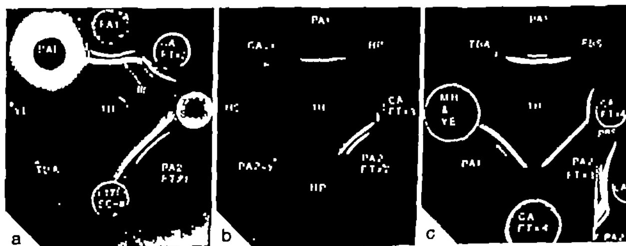


Fig 2 Specificity testing of antifimbrial antibody The 1H antiserum was used. Fig. 2a shows the results with blood agar plate extract (BAE) yeast extract (YE) and tryptose blood agar extract (TBA), compared with purified antigen 1 (PA1) and 2 (PA2), crude antigen (CA) and whole-cell antigens in TA buffer pH 7.5 freeze-thawed four times, of *Moraxella nonliquefaciens 7784 SC-c and of Neisseria elongata* subsp. *glycolytica* 6171/75 SC-a. All antigens, except PA1 and PA2, were filled into the wells several times In Fig. 2b human plasma (HP), and a human serum pool (HS) are compared (at a later date) with the same PA1 PA2, and CA antigens as above, as well as with control antigens, CAcx and PA2cy *Fig. 2c also shows* repeated experiments with the same PA2 and CA antigens, and, in addition, TBA and a mixture of YE and Mueller Hinton broth extract (MH) Larger wells were used for this mixture and for one experiment with an increased amount of CA. The latter well was placed more peripherally than the others. Antigens were stored frozen and thawed (FT) several times (× 1 × 2, × 3 × 4) as indicated One of the wells was filled with phosphate buffered saline (PBS). The inset shows the right side pattern after tannic acid intensification (see the text and Fig 4). The precipitates seen with PA1 are labelled *I and II in Fig. 2a* A weak line to antiserum 1H, seen as heterogeneity *in II,* is indicated with an arrow and III

SC-a). The I precipitate often showed a slight curvature towards adjacent wells regardless of whether these were filled with PBS, TBA or MH & YE (Fig. 2c) This reaction is considered unspecific or insignificant when not continued in front of the well A double I line in Fig 2b, lower right, might be an artefact caused by applying the antigen twice or due to excess antibody

The PA2 preparations, which were obtained by simplifying the preparative procedures to a minimum, were comparable to PA1 in some tests (Fig. 2), but gave only faint precipitates in others (Fig. 3a), as did fresh CA preparations (Fig. 3b-d) It was detected that repeated freeze-thawing in the presence of 1 M KBr could be used to increase the I precipitate (Fig. 3b). A weak intermediate line

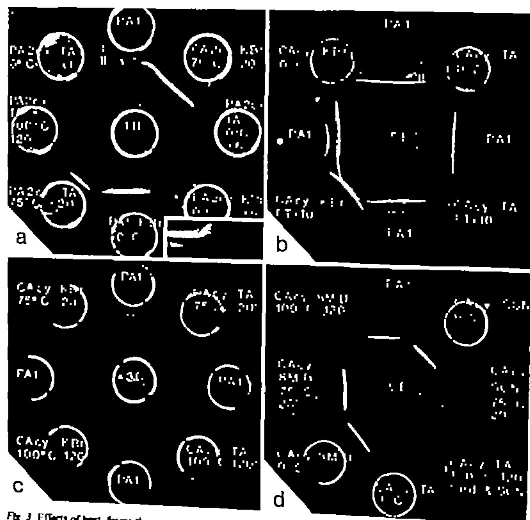


Fig 3 Effects of heat, freeze-thaw treatment, and KBr urea and thiocyanate ions on fimbrial antigens. The antigens, sera and immunoprecipitates are labelled as in Fig and explained in the text. A weak line intermediate between I and II often seen with the SE antiserum, is indicated by an arrow in Fig 3b Mixtures of antigens with various chemicals and subsequent treatments are indicated on the figure TA: 0 01 M tris, 0 01 M NaN_3 buffer pH 7.5 KBr 1 M final concentration in TA buffer pH 7 5 8MU 8 M urea in distilled water SCN 3 M sodium thiocyanate in 0 01 M sodium phosphate buffer pH 6 5 ×6 approximately six times larger volume of antigen in the well (several applications) than in the one labelled ×1 75°C 20' heat treatment of the antigen at 75°C for 20 min, 100°C 120' the same at 100°C for 120 min, FT 10 freeze-thaw treatment in 10 cycles; Sed & SCN the sediment collected from CAcy heat-treated in TA buffer at 100°C for 120 min was suspended in the SCN solution. The inset in Fig. 3a shows part of the lower right-hand immunoprecipitate after tannic acid treatment (see Fig 4).

Electron microscopy of formalin-treated PA1 antigen, which (in several lots) was the only antigen used for immunization, shows a meshwork of fimbrial threads lying straight or slightly curved (Fig. 1a). The diameter of the fimbriae ranges from 6 to 8 nm when filaments running in parallel are measured (4). The width of the light zone is about 4 nm. Very few ends can be seen, but a few rounded structures of various sizes, presumably derived from the outer membrane (*cf.*, ref. 15) are evident (arrows). The PA2 preparation shown in Fig. 1b seems to resemble PA1 in all respects. The crude antigen (CA) definitely contains more of the round structures (Fig. 1c) and is mixed with some whole cells. This contamination may be *reflected* in the yields (see above).

PA2cx was also prepared from *M. nonliquefaciens* 3067/66 SC-a and gave 0.53 per cent of rather impure fimbriae as compared to similar 7784 SC-c preparations. When the same method was applied to the N-a variant of 3067/66 the yield was 0.32 per cent, and subcellular material with no fimbriae was seen by electron microscopy. This latter antigen was used as a fimbria-free control antigen in some tests.

Double immunodiffusion with M. nonliquefaciens 7784 SC-c antigen. The antibody response to formalinized PA1 was studied by immunodiffusion. The preimmune sera showed no activity to purified fimbriae, but after immunization three precipitates were detected (Fig. 2a and b, top and lower right). With all rabbit antisera, both PA1 and PA2 gave rise to two main precipitate lines, I and II, and the *1H* antiserum showed a third less distinct line, III, marked with an arrow on the antiserum side of the precipitate labelled II. Immunoprecipitates were also demonstrated with CA preparations, and there seemed to be fusion of CA lines with PA1 and PA2 lines as seen on Fig. 2a, b and c. Faint precipitates were found with the untreated whole-cell extract of *M. nonliquefaciens* 7784 SC-c, the source of the fimbrial antigen used for immunization. Also antigen prepared from cells grown on Mueller Hinton plates and washed (PA2cy) gave a similar response as PA2. The antisera showed no activity with the blood agar extract (BAE), yeast extract (YE), Mueller Hinton broth extract (MH), tryptose blood agar base (TBA), human serum (HS), human plasma (HP) or the untreated whole-cell antigen from a distantly related, fimbriated bacterium (6171

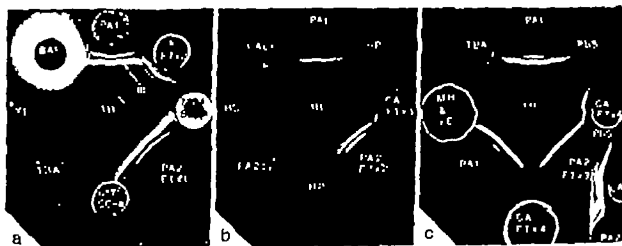


Fig. 2. Specificity testing of antifimbrial antibody. The *1H* antiserum was used. Fig. 2a shows the results with blood agar plate extract (BAE), yeast extract (YE), and tryptose blood agar extract (TBA), compared with purified antigen 1 (PA1) and 2 (PA2), crude antigen (CA), and whole-cell antigens in TA buffer, pH 7.5, freeze-thawed four times, of *Moraxella nonliquefaciens* 7784 SC-c and of *Neisseria elongata* subsp. *glycolytica* 6171/75 SC-a. All antigens, except PA1 and PA2, were filled into the wells several times. In Fig. 2b, human plasma (HP), and a human serum pool (HS) are compared (at a later date) with the same PA1, PA2, and CA antigens as above, as well as with control antigens, CAcx and PA2cy. Fig. 2c also shows repeated experiments with the same PA2 and CA antigens, and, in addition TBA and a mixture of YE and Mueller Hinton broth extract (MH). Larger wells were used for this mixture and for one experiment with an increased amount of CA. The latter well was placed more peripherally than the others. Antigens were stored frozen and thawed (FT) several times (×1, ×2, ×3, ×4) as indicated. One of the wells was filled with phosphate buffered saline (PBS). The inset shows the right side pattern after tannic acid intensification (see the text and Fig. 4). The precipitates seen with PA1 are labelled I and II in Fig. 2a. A weak line to antiserum *1H*, seen as heterogeneity in II, is indicated with an arrow and III.

SC-a). The I precipitate often showed a slight curvature towards adjacent wells regardless of whether these were filled with PBS, TBA or MH & YE (Fig. 2c). This reaction is considered unspecific or insignificant when not continued in front of the well. A double I line in Fig. 2b, lower right, might be an artefact caused by applying the antigen twice or due to excess antibody.

The PA2 preparations, which were obtained by simplifying the preparative procedures to a minimum, were comparable to PA1 in some tests (Fig. 2), but gave only faint precipitates in others (Fig. 3a), as did fresh CA preparations (Fig. 3b-d). It was detected that repeated freeze-thawing in the presence of 1 M KBr could be used to increase the I precipitate (Fig. 3b). A weak intermediate line

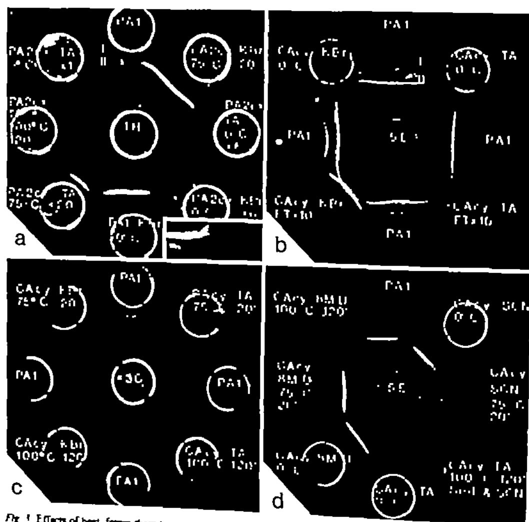


Fig. 3 Effects of heat, freeze-thaw treatment, and KBr, urea and thiocyanate ions on fimbrial antigens. The antigens, sera and immunoprecipitates are labelled as in Fig. 2 and explained in the text. A weak line intermediate between I and II, often seen with the 5E antiserum, is indicated by an arrow in Fig. 3b. Mixtures of antigens with various chemicals and subsequent treatments are indicated on the figure. TA: 0.01 M tris, 0.01 M NaN_3 buffer pH 7.5; KBr: 1 M final concentration in TA buffer pH 7.5; 8MU: 8 M urea in distilled water; SCN: 3 M sodium thiocyanate in 0.01 M sodium phosphate buffer pH 6.5; ×6: approximately six times larger volume of antigen in the well (several applications) than in the one labelled ×1; 75°C 20′: heat treatment of the antigen at 75°C for 20 min; 100°C 120′: the same at 100°C for 120 min; FT×10: freeze-thaw treatment in 10 cycles; Sed & SCN: the sediment collected from CAcy heat-treated in TA buffer at 100°C for 120 min was suspended in the SCN solution. The inset in Fig. 3a shows part of the lower right-hand immunoprecipitate after tannic acid treatment (see Fig. 4).

(arrow) of PA1 to antiserum 5E can be seen. The 3C antiserum shows only the two main precipitates (Fig 3c) Other chaotropic agents like 8 M urea and 3 M sodium thiocyanate, gave predominantly the II precipitate, and this was also the main one seen after 75°C treatment in buffer or in 1 M KBr (Fig 3a, c and d) Heating at 100°C for 120 min gave coagulation and abolished the immunoprecipitation in the gel (Fig. 3c). Thiocyanate was unable to solubilize this material (Fig. 3d).

Electron microscopy showed that the freeze-thawed PA2cx antigen (Fig. 1d) was similar to untreated antigens, but the filaments seemed to be more irregular in outline and tended less to aggregate side by side. On the other hand, Fig 1e clearly shows that PA2cx heat-treated in KBr had a different morphology very few intact fimbriae (arrowheads) can be seen on a background of small rods about 10 nm thick, rod length varies between 20 and 300 nm. Thus, the appearance of the fimbriae is changed after heating in the presence of KBr Immunodiffusion experiments with the antigens after ultracentrifugation showed that the heat treated antigen gave a reaction of identity with a II precipitate, and the freeze-thawed antigen gave a precipitate similar to I but it was broader

Reactions with antigens from other bacteria and the tannic acid intensification of immunoprecipitates. Because only *M nonliquefaciens* 7784 SC-c fimbriae had been used for immunization, it was thought that some of the precipitates might represent strain-specific reactions. For the control experiments, selected variants of closely and more distantly related bacteria were studied

When the antisera were tested with several whole-cell extracts, a weak precipitation occurred initially only with the 7784 SC-c extract, the source of the PA1 fimbriae (*cf* Fig 2a) For the control experiments, the antigens were subjected to freeze-thawing in 1 M KBr (Fig. 4a) and to 75°C for 20 min in 1 M KBr (Fig 4b), since the 7784 SC-c CA antigen precipitates were demonstrated regularly under these conditions. Fig. 4a shows precipitates only with PA1 and 7784 SC-c whole-cell extract. In Fig 4b where tannic acid treatment was used, two immunoprecipitates also with *M nonliquefaciens* 4663/62 SC-a can be seen, whereas 3067/66 SC a is difficult to evaluate due to its location (see also Fig. 5a).

Fig. 4c shows that no precipitate was found with the 3067/66 N-a strain, even when tannic acid was used, thus providing further evidence that it was a fimbrial immunoprecipitate which was being studied. Slight precipitation was occasionally observed with the *M bovis* 10900 fim whole-cell extract. Another lot of PA1 antigen, PA1 was similar to PA1 and gave one line that was shorter towards 10900 fim (Fig. 4c)

The weak precipitates seen with 4663/62 SC-a and 3067/66 SC-a (Fig 4b, but see also Fig. 5a) and, in particular the one seen with *M bovis* 10900 fim (Fig. 4c), seem to resemble the II or the weak intermediate precipitate line (arrow) seen with the 5E antiserum In another control experiment, crude and purified antigens from *M nonliquefaciens* 3067/66 N-a and SC-a were prepared with the same technique as used for 7784 SC-c. Fig 5a shows two precipitate lines to antiserum 1H with a PA2cx preparation of 3067/66 SC-a freeze-thawed in KBr There is fusion of one line with the III line

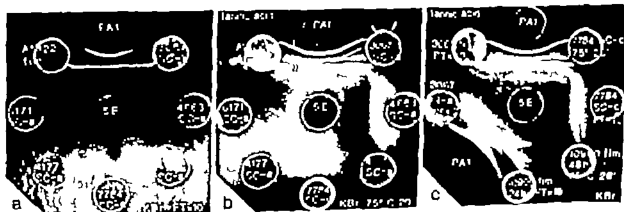


Fig 4 Comparison of purified fimbrial antigen (PA1) with various whole-cell extracts The sera, antigens and immunoprecipitates are labelled as in Figs 2 and 3 The whole-cell antigens tested are labelled with strain/variant designation (see text for species). Treatment applied to all antigens except PA1 is indicated (lower right) on the individual figures. Two gel diffusion plates were intensified with tannic acid as indicated (upper left)

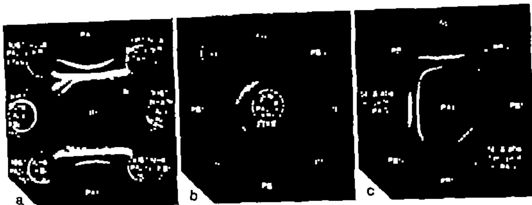


Fig. 5. Experiments with antigens from *Moraxella nonliquefaciens* 3067/66 and absorption of antiserum against strain 7784 SC-c fimbriae with denatured fimbrial protein. Fig. 5a shows experiments with 3067/66 N-a and SC-a antigens, in 1 M KBr purified by the same procedures as used for 7784 SC-a, CAcx and PA2cx antigens. CAcx antigens from the 3067/66 N-a and SC-a variants (middle wells) were kept at 0°C, and PA2cx antigens from the same variants were either freeze-thawed 10 times (upper side wells) or heated at 75°C for 20 min (lower side wells). PA1 (from 7784 SC-c), untreated, was added to the uppermost and the lowermost wells for comparison. In Fig. 5b PA2cx from 3067/66 SC-a, in 1 M KBr freeze-thawed 10 times is tested against various antisera to PA1. Antisera from different rabbits are separated by wells filled with PBS. See text and Figs. 2 and 3 for experimental details and labelling. Fig. 5c shows the result of absorbing the 5E antiserum with fimbrial subunit protein from *M. nonliquefaciens* 7784 SC-c fixed in polyacrylamide gel (abs. fim. prot. in PAG); for control, a parallel absorption with only polyacrylamide gel (abs. no prot. in PAG). PA1 antigen from the same strain was placed in the central well, unabsorbed serum for comparison in the upper well, and PBS in the unused wells. All the plates were intensified with tannic acid.

given by PA1 and partial fusion (spurring) of the other with the II line of PA1. Antiserum 5E (not shown) gave a similar picture, *i.e.* spurring with II (irregularly) and (partial?) fusion with the intermediate line (indicated with an arrow in Figs. 3b and 4b). Fig. 5a also shows that the PA2cx 3067/66 SC-a antigen heat-treated in KBr gave weaker precipitate(s) with the 1H antiserum than that treated by freeze-thawing in KBr. The 3067/66 N-a preparations were negative. The 3067/66 SC-a CAcx antigen in KBr gave no precipitate when untreated, but similar reactions as PA2cx preparations when treated identically. Three to five times more 3067/66 SC-a antigen protein as compared to 7784 SC-c was required to give equally strong immunoprecipitates. In Fig. 5b the PA2cx antigen of 3067/66 SC-a freeze-thawed in KBr shows identical precipitates with the 5E, 5H and 3F antisera. The 1D and 1H antisera also showed this activity (weakly), but in addition showed a more distinct precipitate closer to the antiserum wells, which presumably represents the activity indicated by III (Figs. 2a and 5a).

Since the results with the 3067/66 strain became so much clearer after preparation of PA2cx and CAcx antigens, I checked whether the presence of whole cells might be detrimental to immunoprecipitation. However, there was no effect of adding 3067/66 N-a cells to the PA1 antigen.

It was also thought that the weak precipitates with whole-cell antigens could have been due to a lack of homogenization. The effect of ultrasonication of 7784 SC-c and 3067/66 SC-a cells was tested in 1 M KBr in TA buffer pH 7.5. No significant difference was seen as compared with cells treated by freeze-thawing in 1 M KBr.

Absorption experiments. The antiserum 5E was absorbed with an immunosorbent made from the fimbrial protein band in polyacrylamide gel (16), with the result seen in Fig. 5c. The II immunoprecipitate has disappeared almost completely after the specific protein absorption, whereas no such influence is seen on the serum aliquot absorbed with a similar amount of polyacrylamide gel. The I line seems almost unchanged, but was more clearly weakened, in other experiments. Absorption with the material which coagulated by boiling a PA2cx preparation also removed the II precipitate preferentially from 1H and 5E antisera in further experiments. Absorption with untreated 7784 SC-c fimbriae (PA2cx) removed all precipitating activity from the antisera.

DISCUSSION

Detection of M nonliquefaciens *fimbrial antigens by double immunodiffusion.* Immunization of three rabbits with the most highly purified fimbrial antigen (PA1) of *M nonliquefaciens* 7784 SC-c available gave rise to antibodies that could be detected in gel diffusion against the immunizing antigen. The antigen was pure in SDS polyacrylamide slab gel electrophoresis and in the amino acid sequenator (16) Morphologically slight contamination with subcellular particles and a few whole cells could be detected. Agar plate control antigens were completely negative.

No or only faint precipitates were detectable with some of the 7784 SC-c fimbrial preparations and whole-cell antigens, unless they were subjected to prolonged preparatory procedures or certain types of pretreatment. Freeze-thawing in TA buffer but more effectively in 1 M KBr showed regularly that the antigen formed two main immunoprecipitates (I and II). After heating the antigen at 75°C for 20 min in 1 M KBr mainly the one labelled II (closest to the antiserum well) occurred, and both disappeared by coagulation at 100°C. Heterogeneity in the II precipitate was noted with the 1H antiserum as two closely positioned lines. The weak line on the antiserum side was defined as the III precipitate. A weak precipitate (arrow) was seen between the two main precipitates with the 5E antiserum Serological identity between these two weak lines was not established.

Absorption experiments with fimbrial protein immobilized in polyacrylamide gel, and with heat coagulated fimbriae showed that activity could be removed corresponding to the II precipitate. Precipitates partly fusing with II and completely with III were also given by purified antigen from *M nonliquefaciens* 3067/66 SC-a and the 1H antiserum, whereas the 3067/66 N-a variant preparations were completely negative.

It is concluded therefore, that antibodies to *M nonliquefaciens* fimbrial antigen are present in the antisera, and that the antigen in its most native and polymerized form is almost undetectable by immunodiffusion analysis owing to its filamentous nature or its aggregation tendency or both Furthermore it is suggested that various physical and chemical treatments may change this antigen into diffusible forms some of which are showing immunological cross reactions with antigens from related, fimbriated bacteria It is also possible that one of these forms (I) may change into the others (II/III) The fimbrial antigen is sensitive to prolonged boiling coagulation was seen to occur The experiments with 3067/66 N-a gave no indication of precipitins to cell wall antigens in common with this strain in any of the antisera.

Cross reacting fimbrial antigens. The antisera also reacted with pretreated whole-cell extracts of *M nonliquefaciens* 3067/66 SC-a, 4663/62 SC-a and weakly with *M bovis* 10900 fim but not with extracts from 3067/66 N a (non-fimbriated) or other fimbriated bacteria included in this study (*M atlantae K kingae* and *N elongata* subsp *glycolytica*). It should be noted that *M bovis* is closely related genetically to *M nonliquefaciens* (2 20 21), although only one of two strains gave a positive reaction. *M atlantae* is more distantly related (8), and *K kingae* and *N elongata* subsp *glycolytica* are clearly separated from *Moraxella* genetically (7 21 22 23) The antibodies thus seemed to react with fimbrial antigen of *M nonliquefaciens* 7784 SC-c and closely related bacteria only

However the other whole-cell extracts of fimbriated bacteria giving negative reactions may contain the corresponding antigen in amounts too small to be detected in immunodiffusion as performed here. *M bovis* 4 SC-a may be an example. The yield of PA2cx antigen from 3067/66 SC-a, *e g* was only about one-fifth of PA2 from 7784 SC-c. Moreover the cross-reacting antigens might give weaker reactions in relation to the protein content than those used for immunization. This seemed to be the case with the 3067/66 SC-a antigens.

The experiments with 3067/66 SC-a preparations confirmed that pretreatment is required to reveal precipitates. The results with 4663/62 SC a and *M bovis* 10900 fim indicate that also these strains have antigenic determinants in common with 7784 SC-c and 3067/66 SC-a. Purification of fimbriae, or at least preparation of crude antigen from the other species, will be required to confirm the preliminary findings shown in Fig. 4a and b and to test further for cross reactions.

Bøvre et al (4) studied bacterial agglutination of *M nonliquefaciens* 3067/66 N a and SC a cells with antisera to whole cells and heat-treated cells of both variants. Both identical heat stable and heat labile envelope antigens were detected in the two variants Absorption experiments with the sera indicated as the only clear serological difference that the SC cells were distinctly prone to incomplete agglutination in SC antisera absorbed with N cells It was suggested that this was due to the fimbriae which were probably present in unequal quantities among the cells of the SC population.

The experiments by *Haug & Henriksen* (20) indicated cross-reactions between all the strains of *M lacunata*, *M bovis* and *M nonliquefaciens* studied by them in immunodiffusion, which is in good agreement with the view that the organisms

are closely related. They also found numerous cross-reactions that were difficult to explain. Their sera were obtained by immunizing with a mixture of an ultrasonicated extract and whole cells of unknown fimbriation type, and it cannot be stated with certainty whether the cross-reacting antigens represented fimbriae.

Immunodiffusion studies of fimbrial antigens in other bacteria. Purified fimbriae from *N. gonorrhoeae* F62, colonial type T1 gave one band midway between the antigen and antiserum wells, showing a reaction of identity with a band of the same location from sonically treated cells, regardless of whether the antisera were made from purified fimbriae or from whole-cell extracts. Colonial type T4 (non-fimbriated) gonococcal extracts gave no immunoprecipitate, and antisera produced with T4 type cells did not react with isolated fimbriae (29). Fimbriae from four different gonococcal strains were antigenically distinguishable in immunodiffusion, even though they shared some antigenic determinants, as shown by spurring of lines (10). Single precipitation lines have been detected with fimbrial antigens and antifimbrial sera in *Aeromonas hydrophila* (26), *Pseudomonas aeruginosa* (17) and *E. coli*. The K88 (35) and the K99 (24) antigens of *E. coli* formed precipitates close to the antigen well.

Fimbriae from representative strains of three serotypes of Corynebacterium renale were studied by immunodiffusion. It was found that the fimbriae of one strain were serologically identical with those of other strains of the same serotype and were different from those of strains belonging to the two other serotypes. Fimbriae were also shown to change on sonication but still showed partial cross-reaction with purified fimbrial antigen. After heat treatment, the ability to form immunoprecipitates was increased and shorter pieces were observed by electron microscopy (36). Non-fusing precipitates were formed after chemical treatment. Heterogeneity in the precipitation pattern with crude fimbrial antigen was interpreted as cell wall contamination (25).

The results reported here might facilitate immunological analysis of other fimbrial systems by pointing out the effects of controlled denaturation by KBr, heat, urea or thiocyanate ions. The ease with which extensively purified fimbriae (PA1) are detected by immunodiffusion may be due to slight denaturation taking place during purification.

The technical assistance of Miss *Sissel Dyrnes*, Mrs *Vesleøy Fredriksen*, Mrs *Karin Skarg* and Mrs. *Line Henriksen* is gratefully acknowledged. Professor *Kjell Bøvre* provided and checked most of the bacterial strains used, read the manuscript and also stimulated the work by numerous discussions. *Donna Wiger* M. Sc. read parts of the manuscript to improve it linguistically and pointed out where certain controls were required

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ACID-SOLUBLE NUCLEOTIDES OF *ACHOLEPLASMA (MYCOPLASMA) LAIDLAWII A*

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Virkola, P Acid-soluble nucleotides of *Acholeplasma (Mycoplasma) laidlawii A* Acta path microbiol. scand Sect. B, 86 179–183 1978

A study was made of the acid-soluble nucleotides present in *Acholeplasma (Mycoplasma) laidlawii A* during growth The nucleotide pool of this organism is meagre, both qualitatively and quantitatively The main nucleotides are uridine mono- and diphosphates, and none of the more complex nucleotide compounds, such as uridine diphosphate (UDP) sugars or sugar peptides, required for cell wall formation, were detected The nucleotide composition of *A laidlawii A* is compared with that of the *Streptococcus* L form

Key words. Acholeplasma (Mycoplasma) laidlawii A, acid-soluble nucleotides

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The structural integrity of the bacterial cell is maintained by a peptidoglycan present in the cell walls In *Streptococcus pyogenes* (*Edwards & Panos* 1962), *Staphylococcus aureus* (Fodor & Tóth 1965 *Fodor* 1966) and *Bacillus licheniformis* and *B subtilis* (*Ward* 1975), stable L-phase variants, although devoid of an organized cell wall, accumulate nucleotide precursors of peptidoglycan, UDP N-acetyl muramic acid and UDP-N-acetyl muramylpeptides during growth

Cells of *Mycoplasma* and *Acholeplasma* are also surrounded by a membrane only and thus resemble the L-phase variants of bacteria in size and morphology Although the mycoplasmas do not contain either diaminopimelic or muramic acid or their macrocomplex, they do contain glucosamine (*Razin* 1963) and galactosamine (*Engelman & Morowitz* 1968) in their membranes Several unidentified strains from goats, sheep and poultry as well as *Acholeplasma laidlawii*, were found to contain trace amounts of neutral sugars extractable with hot aqueous phenol (*Plackett et al* 1963). No amino sugars were detected in these preparations From *A laidlawii* a polymer composed of N-acetyl glucosamine has been isolated and characterized (*Gilliam & Morowitz* 1972). This appears to be weakly associated with the cytoplasmic membrane (*Terry & Zupnik* 1973). Lipopolysaccharides isolated from *A laidlawii* contain both neutral (glucose, galactose and mannose) and N-acylated (fucosamine, an unidentified deoxyhexosamine, galactosamine and glucosamine) sugars (*Smith et al.* 1976)

No information was available as to whether mycoplasmas contain the nucleotide precursors of the peptidoglycan necessary for cell wall formation A study was made, therefore, of the acid-soluble free nucleotides in *A laidlawii A*

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade The nucleotide standard solutions were purchased from Calbiochem, Lucerne, Switzerland

Organism

Acholeplasma (Mycoplasma) laidlawii A was kindly supplied by *E A Freundt*, Institute of General Pathology and Bacteriology University of Aarhus, Denmark.

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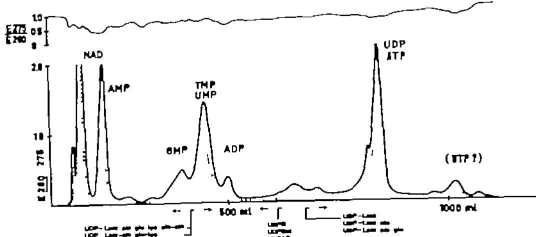


Fig 1 Acid-soluble free nucleotides of *Acholeplasma (Mycoplasma) laidlawii A* after 24 hours' growth (15 l culture).

UDPAG = Uridine-5'-diphosphate N-acetyl-glucosamine

UDPAG-Lact = Uridine-5-diphosphate acetylmuramic acid
UDPAG-Lact, ala
UDPAG-Lact, ala, glu
UDPAG-Lact, ala, glu, lys,
UDPAG-Lact, ala, glu, lys, ala, ala } Uridine-5-diphosphate acetylmuramic acid peptides
UDPG = Uridine-5'-diphosphate glucose
UDPGal = Uridine 5'-diphosphate galactose

the cells. It contained mainly NAD AMP GMP and TMP with small amounts of UMP Since no significant change in the nucleotide pattern occurred during culture, the presence of these nucleotides in the culture medium did not restrict the growth of *A laidlawii A* However it was not possible to estimate whether the nucleotides found in the cells had been synthesized by them or taken up from the culture medium

The requirements of *A laidlawii A* for nucleic acid precursors have been studied by *Razin & Knight* (1960), *Razin* (1962) and *Lluka & Smith* (1974), who found that the only nucleic acid precursors necessary were guanine, thymine and either cytosine or uracil.

Accordingly we conclude that *A laidlawii A* is able to synthesize ADP ATP and UDP that its free nucleotide pool is small, and that it apparently lacks

TABLE 1 *Extractable Acid-soluble Ultraviolet-absorbing Material*

	A laidlawii A OD total[a]		*Streptococcus*[b] OD total[a]	L form[b] OD total[a]
	/1000 ml medium	/g dry cells	/g dry cells	/g dry cells
	SD	SD		
total	62 ± 24	246 ± 94	469	594
non-polar material	18 ± 11	71 ± 44	58	13
nucleotide content	44 ± 21	175 ± 83	411	581

[a] Expressed as total absorbance (OD total)·
(OD₂₆₀ of perchloric acid extract) x (volume of extract in ml), average of six determinations (*Franzen & Binkley* 1961)

[b] *Edwards & Panos* (1962), standard deviations not given

Cultural Conditions

The *Butler & Knight* (1960) modification of the original *Edward* medium (1947) was used with the following changes. heart infusion to 1 per cent peptone broth (Evans), 20 vol of horse serum to 10 vol of human serum (inactivated by heating for 30 min at 56°C), the concentration of penicillin G (when used) in the final medium was 200 U/l In addition, the medium contained 7 5 g/l glucose (*Razin* 1964).

The inoculum (0.5 per cent) used was a 24-hour-old *A laidlawii* culture suspension, and the organisms were grown at 37°C in 5–10 l culture flasks with three different degrees of aeration (*Virkola* 1972) Growth was expressed. (1) as turbidity units measured by a Klett Summerson colorimeter (filter S 54), (2) as living cells estimated by the colony count method for mycoplasma (*Butler & Knight* 1960). After 10–48 hours growth, the culture was chilled rapidly (*Saukkonen & Virkola* 1963) and the cells were harvested by centrifugation (13200 × g 20 min, 50 ml batches) in cold. (Attempts to collect cells of *A laidlawii A* by the continuous flow system of a Servall SS centrifuge were unsuccessful). The combined pellets were washed three times with three volumes of 0 9 per cent NaCl and frozen Blood agar plates were used to confirm the absence of bacterial contamination.

Column Chromatography

The acid-soluble nucleotides were separated as before (*Virkola* 1970) using Dowex 2 resin, HCOO-form, 200–400 mesh, 8 per cent cross-linked packed in columns 12–20 cm long and 0 8–1 0 cm in diameter The columns were run at room temperature or at + 2°C with a cooling jacket round the column (*Virkola* 1969). The eluate was collected in 5 ml fractions The appearance of nucleotides in the eluate was monitored as absorbance at 260 nm and at 275 nm The detection limit of this system was about 25–100 nmol/nucleotide (*Virkola* 1970). The fractions of each peak were combined and lyophilized During freeze-drying, the removal of ammonium formate was enhanced by gentle heating when necessary

Paper and Thin layer Chromatography

The individual nucleotide peaks in column chromatography were identified by paper and thin-layer chromatography The separated nucleotides were also hydrolyzed with HCl and the resulting bases analyzed by spectrophotometry (*Saukkonen* 1956) The nucleotides and bases were visualized and their positions marked under UV light The solvents used in paper chromatography were. for nucleotides isobutyric acid – ammonia (*Magasanik et al* 1950) neutral ethanol – ammonium acetate – water (*Paladini & Leloir* 1952) propanol – ammonia – water (*Baddiley et al* 1956); for amino sugars and acids n butanol – acetic acid – water (*Strange & Dark* 1965) After acid hydrolysis of the nucleotides, purine and pyrimidine bases were separated by paper chromatography with 86 per cent n-butanol as solvent (*Markham & Smith* 1949)

The nucleotides were also separated by thin layer chromatography according to *Randerath & Randerath* (1964 a, b)

An Auto-Analyzer method (*Henriksen* 1965) was used for phosphate determinations.

RESULTS AND DISCUSSION

Washing of the cell mass is a critical step in the analysis Extensive washing tends to disrupt the cells, but washing is essential to remove the growth medium with its contaminating nucleotides. We used absorbance at 260 nm to observe the amount of acid-soluble material in successive washings. The material decreased rapidly during the first three washings and then remained at a constant level Three washings of the pellet were therefore considered to be optimal.

The organisms were harvested at intervals during the logarithmic phase of growth In the first interval (10–16 hours) the yield of organisms was so small that the different nucleotides could not be measured. In later intervals the nucleotide pool (Fig 1) was shown to contain eight nucleotides, some of which are found in the *Streptococcus* L-phase (*Edwards & Panos* 1962). However there were also several differences. None of the peaks contained any amino acids after acid hydrolysis as tested by paper chromatography The fractions in which nucleotide sugar derivatives were most likely to appear (550–700 ml) did not contain any sugars when tested by paper chromatography Thus *A laidlawii A* seems to lack several nucleotide precursors of the cell wall constituents, UDP N-acetylglucosamine, UDP-N hexosamine and UDP muramyl peptides which are present in the *Streptococcus* L form (see also Table 2) However unlike the latter the mycoplasma contains UMP and TMP In growing *A laidlawii A* the total amount of free nucleotides is smaller than in *Streptococcus* or its L form (Table 1). *A laidlawii A* also contains smaller total amounts of extractable acid-soluble ultraviolet-absorbing material and free nucleotides, but a larger relative amount of non-polar material

Quantitative analysis showed that the dominating nucleotides in *A laidlawii A* were UDP and UMP (Table 2) The corresponding analysis of the *Streptococcus* L form (*Edwards & Panos* 1962) showed clearly that the dominant nucleotides in their pool were those concerned in the more advanced steps of cell wall synthesis (UDP muramyl peptide, UDP N AG UDP-hexosamine)

No change in the composition of the free nucleotides of *A laidlawii A* was produced by the temperature during the separation the addition of penicillin to the growth medium or the degree of aeration during growth

Both before and after culture of mycoplasma, the culture medium was analysed in the same way as

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TABLE 2. *Nucleotide composition of* A. laidlawii A, Streptococcus *and the L form*[a] *of the latter*

	A laidlawii A[b]		*Streptococcus*[a]	L form[a]
	µmol/1000 ml medium	µmol/g	µmol/g	µmol/g
CMP	–	–	2.14	2.13
DPN	not measured		1 75	2.61
AMP	0 16	0 66	0 55	0 87
GMP	*0 15*	*0.50*	not identified	
UMP TMP	0 25	0 98	3 66	–
UDP mur-peptide	–	–	–	8 81
Uridine nucleotide	–	–	2 07	–
ADP	0 09	0 37	0 46	0 48
UDP N AG	–	–	2 03	4 21
UDP-hexosamine	–	–	0 88	1 12
GDP	–	–	1 27	1 79
UDP	0 68	2 71	1 45	1 61
ATP	0 08	0 33	1 12	1 90

a) *Edwards & Panos* (1962)
b) *average of three determinations*

nucleotide peptides. The L-phase variants of different species of bacteria have been classified into three groups on the basis of the type of defect in peptidoglycan synthesis (*Ward* 1975). *Plapp & Kandler* (1964) suggested that the presence of alanine racemase could be used for differentiating between mycoplasmas and L-phase micro-organisms. According to our results, mycoplasmas differ from the L forms of bacteria in that they apparently lack the nucleotide precursors needed for cell wall formation. This might serve as one more criterion for classification.

The author wishes to thank Prof. *V Kauppinen*, Ph D., Helsinki University of Technology Dept. of Chemistry and Prof *J J Saukkonen*, M D., Central Public Health Laboratory for their valuable help and advice, and Mrs. *Sirkka Metiäinen* and Mrs. *Liisa Vuorenpää Karppinen* for their technical assistance. This research was supported by the *Emil Aaltonen Foundation*

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RAPID ONCOGENESIS IN VIVO BY CHICKEN RETROVIRUS OK10

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Hortling, L. Rapid oncogenesis in vivo by chicken retrovirus OK10. Acta path. microbiol. scand. Sect. B, 86: 185–192, 1978.

The OK10 virus complex is known to contain two detectable viruses: a) focus-forming virus OK10V that transforms chick embryo cells, and b) an associated virus OK10AV present in excess that converts the morphology of cultured chick embryo cells. The pathogenesis of OK10 virus infection was studied, using 2–4 day old Brown Leghorn chickens. A group of chickens was sacrificed at weekly intervals, serum samples were taken and tissues were examined for virus. Autopsies of the chicken were performed and gross and microscopic changes were registered. After intraperitoneal injection of 10^4 focus-forming units of OK10 virus, infectious OK10AV was detected after one week in Bursa Fabricius, thymus and liver, and OK10V after two weeks in Bursa Fabricius but in no other organ. Neutralizing serum antibodies developed within three weeks. The first malignant changes, in the mesentery, were detected after three weeks. The infection was lethal in all experiments within 6–8 weeks. In the mesentery the tumors consisted of large tumour cells with clear cytoplasm, a large nucleus and prominent nucleoli. The origin of these cells could not be established. The cells were surrounded by lymphoid cells. From the tumours, continuous cell lines were established which produced both viruses OK10V and OK10AV and had blast-like morphology. After intravenous injection of OK10 virus, tumours could also be found in liver, kidneys and testes. The associated virus OK10AV was infectious for chickens and induced neutralizing serum antibodies. One out of seven chickens died of leukosis after 1½ years. The *OK10* virus complex, consisting of a tumour forming and a weakly oncogenic associated virus, appears to have a multiple oncogenic potential in its rapid oncogenic action *in vivo*.

Key words: Avian retrovirus, RNA tumour virus, chicken, oncogenesis, pathogenesis.

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Avian C-type tumour viruses are divided according to their oncogenic potential into leukaemia and sarcoma viruses. This classification has been further extended by *Temin* (33) and *Coffin* (7), who divided RNA tumour viruses into four groups, depending on their transforming interactions with host cells in culture and the type of tumour they induce in animals.

To the first group (7) belong the sarcoma viruses which transform fibroblasts in tissue culture and cause sarcomas in chicken.

The second group consists of viruses that induce leukaemia and similar diseases within a month and transform cells *in vitro*. Strain MC 29 avian leukosis virus (15, 21) causes a broad spectrum of cancers of myeloid origin (18) and transforms chick embryo cells *in vitro* within three days, forming distinct foci in chick embryo cell cultures (4, 16, 17). Avian myeloblastosis virus transforms haematopoietic cells in culture (1, 3, 22, 23). *In vivo* this virus causes myeloblastosis, osteopetrosis, lymphoid leukosis and nephroblastomas in chickens (2). Inoculation of chickens with avian erythroblastosis virus leads to acute erythroleukaemia within a few weeks after infection (10).

In vitro this virus transforms both chicken bone marrow cells and chick embryo fibroblasts (12,13). All these viruses consist of a transforming virus and an associated virus present in excess (14, 19, 24).

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Hortling, L. Rapid oncogenesis in vivo by chicken retrovirus OK10. Acta path. microbiol. scand. Sect. B, *86*: 185–192, 1978.

The OK10 virus complex is known to contain two detectable viruses: a) focus-forming virus OK10V that transforms chick embryo cells, and b) an associated virus OK10AV present in excess that converts the morphology of cultured chick embryo cells. The pathogenesis of OK10 virus infection was studied, using 2–4 day old Brown Leghorn chickens. A group of chickens was sacrificed at weekly intervals, serum samples were taken and tissues were examined for virus. Autopsies of the chickens were performed and gross and microscopic changes were registered. After intraperitoneal injection of 10^4 focus-forming units of OK10 virus, infectious OK10AV was detected after one week in Bursa Fabricius, thymus and liver, and OK10V after two weeks in Bursa Fabricius but in no other organ. Neutralizing serum antibodies developed within three weeks. The first malignant changes, in the mesentery, were detected after three weeks. The infection was lethal in all experiments within 6–8 weeks. In the mesentery, the tumors consisted of large tumour cells with clear cytoplasm, a large nucleus and prominent nucleoli. The origin of these cells could not be established. The cells were surrounded by lymphoid cells. From the tumours, continuous cell lines were established which produced both viruses OK10V and OK10AV and had blast-like morphology. After intravenous injection of OK10 virus, tumours could also be found in liver, kidneys and testes. The associated virus OK10AV was infectious for chickens and induced neutralizing serum antibodies. One out of seven chickens died of leukosis after 1½ years. The OK10 virus complex, consisting of a tumour forming and a weakly oncogenic associated virus, appears to have a multiple oncogenic potential in its rapid oncogenic action *in vivo*.

Key words: Avian retrovirus, RNA tumour virus, chicken, oncogenesis, pathogenesis

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Avian C-type tumour viruses are divided according to their oncogenic potential into leukaemia and sarcoma viruses. This classification has been further extended by *Temin* (33) and *Coffin* (7), who divided RNA tumour viruses into four groups, depending on their transforming interactions with host cells in culture and the type of tumour they induce in animals.

To the first group (7) belong the sarcoma viruses which transform fibroblasts in tissue culture and cause sarcomas in chicken.

The second group consists of viruses that induce leukaemia and similar diseases within a month and transform cells *in vitro*. Strain MC 29 avian leukosis virus (15, 21) causes a broad spectrum of cancers of myeloid origin (18) and transforms chick embryo cells *in vitro* within three days, forming distinct foci in chick embryo cell cultures (4, 16, 17). Avian myeloblastosis virus transforms haematopoietic cells in culture (1, 3, 22, 23). *In vivo* this virus causes myeloblastosis, osteopetrosis, lymphoid leukosis and nephroblastomas in chickens (2). Inoculation of chickens with avian erythroblastosis virus leads to acute erythroleukaemia within a few weeks after infection (*10*).

In vitro, this virus transforms both chicken bone marrow cells and chick embryo fibroblasts (12,13). All these viruses consist of a transforming virus and an associated virus present in excess (14, 19, 24).

TABLE 1 *Detection of OK10 and OK10AV Virus in OK10 Virus Infected Chickens by Virus Assay from Culture Media of Organ Cultures Established from Various Tissues*

Days after infection	Chicken No	Bone marrow		Spleen		Liver		Thymus		Bursa	
		OK10V	OK10AV	OK10V	OK10AV	OK10V	OK10AV	OK10V	OK10AV	OK10V	OK10AV
7	1	–	–	–	–	–	+	–	+	–	+
	2	–	–	–	–	–	+	–	+	–	+
14	3	–	–	–	–	–	+	–	+	+	+
	4	–	–	–	–	–	+	–	+	+	+
21	5	–	–	+	+	+	+	+	+	+	+
	6	–	–	+	+	+	+	+	+	+	+
28	7	–	–	+	+	+	+	+	+	+	+
	8	–	–	+	+	+	+	+	+	+	+

The Assays were performed using combined focus titration (for OK10V) and interference (for OK10AV), see Materials and Methods

RESULTS

Detection of Virus in Chickens Infected with OK10 Virus

The associated virus OK10AV was detected after one week in Bursa Fabricius, thymus and liver but not in spleen and bone marrow. Three weeks after infection, the associated virus could also be detected in the spleen (Table 1).

TABLE 2 *Neutralizing Serum Antibodies in OK10 Virus Infected Chicken*

Days after infection	Chicken No	Neutralization index[a])
–	Control	1.0
7	1	1.07
	2	1.05
14	3	1.05
	4	1.07
21	5	0.61
	6	0.51
28	7	0.31
	8	0.43
35	9	0.07
	10	0.29

a) The neutralization index denotes the ratio of infectious OK10V virus in focus-forming units, detected after treatment with 1:50 dilutions of the test serum and control sera, respectively

The transforming virus OK10V was detected after two weeks in Bursa Fabricius but not in any of the other organs tested. Three weeks after infection OK10V could be detected in all the organs tested except in bone marrow (Table 1).

No virus was detected in the controls.

Neutralizing Antibodies in Chickens Infected with OK10 Virus

Neutralizing serum antibodies against the OK10 virus complex were detected for the first time after three weeks. The titre of neutralizing antibody increased up to the end of the experiment. No serum antibodies could be detected in uninfected chickens (Table 2).

Tumour Formation in Chickens Infected with OK10 Virus

Macroscopic tumours 1–2 mm in size were seen in the mesenterium of the gut after three weeks. Ascites was found at the same time (Table 3). After six weeks the size of the tumours had increased to 5–8 mm, and macroscopic tumours could easily be detected also in the liver (Fig. 1). No tumours were detected in any other organs.

When chickens were injected intravenously with OK10 virus, tumours could be detected also in the liver, kidneys and testes.

Histology of the mesenteric tumours (Fig. 2) showed that they were composed of two kinds of cells, clear cells with a large nucleus, prominent nucleoli and cytoplasmic vacuolation and surrounding lymphocytes. This pattern could be seen three

The third group consist of viruses that are not known to transform any type of cells in culture, but they do induce leukaemia with a long latent period (5 8 27). This group consists of the Rous associated viruses.

The fourth group does not transform cells in culture or is known to cause diseases in chickens (9 26) Only one virus is known for this group viz. RAV-0

OK10 a recently isolated RNA tumour virus (*Oker Blom et al In press*) was originally isolated from an embryo of a naturally infected leukotic chicken. Based on its behaviour *in vitro*, the OK10 virus appears to belong to the second group in the classification mentioned above. It has been shown that OK10 is composed of a transforming virus OK10V and an associated virus OK10AV present in excess. OK10AV like Rous associated viruses, converts the morphology of chick embryo cells *in vitro* during prolonged cultivation (28). In contrast, OK10V induces distinct foci under agar overlay and colonies in soft agar within a few days. Both OK10V and OK10AV seem to belong to subgroup A according to interference and neutralization tests (29) This paper describes the pathogenesis in vivo of the OK10 virus complex

MATERIALS AND METHODS

Chickens

The chicken used were 2-4 days old Brown Leghorn chickens (C/0 or C/E cell type), originating from eggs brought to our institute in 1969 from Houghton Poultry Research Station, Huntingdon, England This flock of chickens has remained leukosis-free as evidenced by continuous RIF (resistance inducing factor) COFAL (complement fixation avian leukosis), radioimmunological and neutralization assays (31)

Viruses and Virus Assays

OK10 virus was isolated from an embryo of a leukotic hen (28) The stock virus for the present experiments was collected form the culture media of secondary chick embryo cells infected with OK10 virus. The preparation had a titre of 5×10^4 focus forming units/ml (OK10V titre) and about 10^7 interfering units per ml (OK10AV titre) The titre of purified OK10AV was 10^4 interfering units per ml and 0 5 ml was used to infect 2-4 day old chickens Purified OK10AV was obtained by diluting out the OK10V virus.

RSV (RAV 1) was originally obtained from Dr H *Hanafusa* Rockefeller University New York, N Y U S A The stock virus used in the interference assay was grown in secondary cultures of chick embryo fibroblasts The interference titration to assay OK10AV virus was performed according to *Rubin* (30) using secondary chick embryo fibroblasts in a dose of 250 focus forming units of RSV (RAV 1) virus per 20 cm^2 culture OK10V virus was titrated by focus formation (*Oker Blom et al in press*)

Infection and Observation of Chickens

A group of ten 2-4 day old chickens was injected intraabdominally with 0 5 ml of the OK10 virus stock. Two chickens were sacrified at weekly intervals, serum was collected and autopsies were performed. Uninfected chickens were sacrificed at the age of three and five weeks to serve as controls Tumour formation was recorded and routine histologic sections were prepared form liver thymus, spleen, bone marrow Bursa Fabricius and tumours.

Another group of seven chickens was injected intra abdominally with 10^4 interfering units of OK10AV

Determination of Neutralizing Serum Antibodies

The neutralization tests were carried out as described previously (28) using a serum dilution of 1:50 and OK10 virus purified by five successive clonings.

Cell Cultures

Main experiment Virus expression from the blood samples was measured by interference titration from buffy coat cultures, using lymphoprep (Nyegaard & Co A/S, Oslo Norway) for the purification of the buffy coat cells. Cell cultures were established from liver thymus, spleen, bone marrow Bursa Fabricius and tumours. The organs were minced into pieces with scissors and by pipetting, and the tissue fragments were seeded into growth medium using medium 199 supplemented with 10% tryptose phosphate broth, 10% foetal calf serum, 200 units/ml penicillin and 200 μg/ml streptomycin.

Second experiment In a separate experiment, continuous cell lines were established from tumours induced by OK10 virus. Three 2-4 day old chickens were infected intraperitoneally with 5×10^4 focus-forming units of OK10V and were sacrified 40 days later Tissue cultures were prepared from the tumours as above.

Third experiment In this experiment one chicken was injected intramuscularly with 10^6 tumour cells from a tumour induced by OK10 virus The chicken was sacrified 40 days later and autopsy was performed The bird had tumours in the kidneys, liver adrenals and testes Histologically the tumour cells in the liver and kidneys were large blast cells. A pool was prepared from the bone marrows of this chicken and from another chicken which had been infected two months earlier with 2×10^5 transformed chicken embryo cells. On autopsy a tumour was found at the right metatarsus. Further large cystic tumours were found in both kidneys and there were also tumours in both testes Histologically the tumours were composed of cartilage, cysts and epithelial like malignant cells A continuous cell line was established from the pooled bone marrows

Growth of Cells in Soft Agar

The method described by *MacPherson* in 1969 (20) was used Transformed cells were grown in 0 3% agar (Bacto Difco Laboratories, Detroit, Michigan) on top of 0 5% agar in petri dishes The number of colonies grown in agar was recorded after 8-10 days

Test for Growth in Low Serum Concentration

In order to compare the growth of the transformed cells in different serum concentrations, the normal concentration 10% was lovered to 0 9%

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Histology of the mesenteric tumours (Fig. 2) showed that they were composed of two kinds of cells, clear cells with a large nucleus, prominent nucleoli and cytoplasmic vacuolation and surrounding lymphocytes. This pattern could be seen three

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Second experiment In a separate experiment, continuous cell lines were established from tumours induced by OK10 virus. Three 2–4 day old chickens were infected *intraperitoneally* with 5×10^4 focus-forming units of OK10V and were sacrified 40 days later Tissue cultures were prepared from the tumours as above

Third experiment In this experiment one chicken was injected intramuscularly with 10^4 tumour cells from a tumour induced by OK10 virus The chicken was sacrified 40 days later and autopsy was performed The bird had tumours in the kidneys, liver adrenals and testes. Histologically the tumour cells in the liver and kidneys were large blast cells A pool was prepared from the bone marrows of this chicken and from another chicken which had been infected two months earlier with 2×10^5 transformed chicken embryo cells On autopsy a tumour was *found at the right metatarsus* Further large cystic tumours were found in both kidneys and there were also tumours in both testes Histologically the tumours were composed of cartilage, cysts and epithelial like malignant cells A continous cell line was established from the pooled bone marrows

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Fig. 7 Tumours in the mesenteries of the gut three weeks after infection with OK10 virus. Haematoxylin-eosin staining. The central area is composed of tumour cells with a large nucleus and prominent nucleoli. Lymphoid cells surrounded the central tumour mass. × 40

B Boundary area between tumour cells (lower left) and lymphoid cells (top right). × 100

C Higher magnification of the indicated area in B. × 400

TABLE 3 *Tumour Formation of OK10 Virus Infected Chicken*

Days after Infection	Chicken No	Malignant changes
7	1	–
	2	–
14	3	–
	4	–
21	5	Mesenterium, liver ascites
	6	Mesenterium, liver ascites
28	7	Mesenterium, liver ascites
	8	Mesenterium, liver ascites
35	9	–
	10	Mesenterium, liver ascites

The chickens were sacrificed at weekly intervals and autopsies were performed.

weeks after infection in all the chickens, except one (chicken 9 in Table 3) which, however formed serum antibodies against OK10 virus (Table 2).



Fig 1 Tumour formation in a 6-week-old chicken injected intraperitoneally when newborn with 500 ul of OK10 virus, titre 5×10^4 FFU/ml. Tumours can be seen in the mesenterium (M) of the gut and in the liver (L). There are no tumours in thymus (T) and Bursa Fabricius (B). The chicken had developed ascites.

Effect of OK10AV Infection in vivo

A group of seven chickens was infected with OK10AV. One bird was sacrified at the age of 1½ months. No macroscopic tumours could be detected. Blood samples were taken at 3, 5 and 16 months. At three months, two out of six chickens had viraemia but no neutralizing serum antibodies. They were possibly tolerant. Later on they both developed antibodies. Two chickens were non-viraemic but had neutralizing serum antibodies, one of them became viraemic at 5 months and both were viraemic at 16 months. The last two chickens were viraemic and had neutralizing serum antibodies all the time.

The amount of neutralizing serum antibodies was dependent on virus expression. In chickens positive for virus in the buffy coat culture at the age of three months, a 40–90% neutralization was observed, while 100% neutralization was observed in chickens with no virus expression. At 5 and 16 months the neutralization of virus by serum ranged from 80–100%.

No difference was observed between leukotic and non-leukotic chickens as regards antibody formation.

After 1½ years, one of the chickens became ill and died. Tumours could be observed macroscopically in the thymus, skin, muscle, liver, mesentery and heart. A tumour was also seen at the site where Bursa Fabricius is normally located. Microscopically malignant changes were observed in cervical, subcutaneous and mesenteric lymph nodes. Metastatic changes were observed in the heart, muscle, bone marrow and liver. The histologic changes were classified as leukosis (Fig 3). Histology revealed tumour tissue in the spleen, lungs and kidneys.

Establishment of Continuous Cell Lines From Tumours Induced by OK10 Virus

In the second experiment (see Materials and Methods), continuous cell lines were established from tumours induced by OK10 virus from two different chickens. No tumours were found in a third chicken.

These cell lines were grown in culture for 5 months, and the cells had blast-like morphology. They grew in clumps in the suspension. In the growth medium of these cultures, the titre of OK10V was about 10^4 focus-forming units per ml and that of OK10AV 10^7 interfering units per ml.

In the third experiment (see Materials and Methods), a continuous cell line was derived from the pooled bone marrows of two moribund OK10 virus-infected chickens. The birds had desseminated tumours at that time. This cell line has been growing continuously in the laboratory for two

results have been confirmed by time lapse photography (*Oker Blom et al* unpublished). Infection of chick embryo cells with the associated virus OK10AV also causes a changed morphology (conversion) of chicken embryo fibroblasts (28). However it is not possible to exclude the possibility (11) that haematopoietic cells in chicken embryo cell cultures could be transformed.

OK10V was found in Bursa Fabricius two weeks after infection and later in spleen, liver and thymus. No malignant changes were found in Bursa Fabricius in chickens infected with the OK10 virus complex when they died at the age of 1–2 months, nor have tumours been described in Bursa Fabricius for any of the other acute leukaemia viruses.

In chickens infected with RAV 1 virus expression was also found at two weeks after infection in Bursa Fabricius (32). The development of a tumour at the site of Bursa Fabricius in one of the OK10AV infected chickens is in agreement with the observation that malignant leukotic changes develop in Bursa Fabricius in RAV 1 infected chickens, where transformed lymphoblasts can be found in lymphoid follicles in Bursa Fabricius eight weeks after infection (8 27) This suggests that OK10AV is a lymphoid leukosis virus like RAV 1

In conclusion, the OK10 virus complex, composed of a rapidly oncogenic virus OK10V and an associated weakly oncogenic virus OK10AV may provide a good tool for studying the mechanism of oncogenesis *in vivo* and *in vitro* and the factors that regulate these processes.

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Fig 3 Liver metastasis in an OK10AV infected chicken which died 17 months after infection. Normal liver cells can be seen in the lower left-hand corner Haematoxylin-eosin staining × 100



years. In the growth medium, the focus-forming titre of OK10V was about 10^4 focus forming units per ml. The cells grew in clumps in the suspension, as shown in Fig. 4 They formed colonies in soft agar and their serum requirement was reduced, they grew well in 0 9% foetal calf serum.

DISCUSSION

The present experiments showed that OK10 virus, a newly isolated retrovirus, induced rapid oncogenesis in 2–4 day old chickens. The malignancy was invariably lethal within two months. The tumours could be detected after three weeks in the mesentery of the gut. At the same time large amounts of ascites had been formed.

When chicken were injected intravenously with OK10 virus, tumours were found also in the liver kidneys and testes, thus showing the wide spectrum of tumourigenicity caused by OK10 virus.

According to *Coffin s* classification (7), the OK10 virus complex belongs to the group of avian retroviruses which induce leukaemia and other malignancies within a month, and which also have another non-transforming associated virus in ex cess. The question of helper dependence of OK10V has not yet been solved.

The primary target cell in OK10 virus oncogenesis is not known. The histology (kindly performed by Dr *L. N Payne* Houghton Poultry Research Station, Huntingdon, England) of the mesenteric tumours resembles that of the MH 2 endothelioma described previously (6) with its large cells with big nucleoli and vacuolated cytoplasm. The surrounding lymphocytes might be a host reaction to the tumour cells or they might be malignant cells. The latter possibility is supported by the fact that continuous cell lines with blast like morphology were established from the tumours. These cell lines had properties of malignant cells, the cells formed colonies in soft agar cultures, and had a low serum dependence.

The OK10 virus has also other properties which suggest that it might belong, along with the MH 2 virus, to the acute leukaemia-carcinoma viruses which are known to cause many different kinds of tumours in the chicken (25).

Like the MC29 avian leukosis virus (4 16 17) and avian erythroblastosis virus (13) which also belong to the acute leukaemia-carcinoma viruses, OK10 virus transforms fibroblasts *in vitro*. These

Fig 4 Cells from a continuous cell line established from the bone marrows of moribund chickens (see Materials and Methods). Haematoxylin-eosin staining. × 188

results have been confirmed by time lapse photography (*Oker Blom et al* unpublished). Infection of chick embryo cells with the associated virus OK10AV also causes a changed morphology (conversion) of chicken embryo fibroblasts (28). However it is not possible to exclude the possibility (11) that haematopoietic cells in chicken embryo cell cultures could be transformed

OK10V was found in Bursa Fabricius two weeks after infection and later in spleen, liver and thymus. No malignant changes were found in Bursa Fabricius in chickens infected with the OK10 virus complex when they died at the age of 1–2 months, nor have tumours been described in Bursa Fabricius for any of the other acute leukaemia viruses.

In chickens infected with RAV 1 virus expression was also found at two weeks after infection in Bursa Fabricius (32) The development of a tumour at the site of Bursa Fabricius in one of the OK10AV infected chickens is in agreement with the observation that malignant leukotic changes develop in Bursa Fabricius in RAV 1 infected chickens, where transformed lymphoblasts can be found in lymphoid follicles in Bursa Fabricius eight weeks after infection (8 27). This suggests that OK10AV is a lymphoid leukosis virus like RAV 1

In conclusion, the OK10 virus complex, composed of a rapidly oncogenic virus OK10V and an associated weakly oncogenic virus OK10AV may provide a good tool for studying the mechanism of oncogenesis *in vivo* and *in vitro* and the factors that regulate these processes.

Thanks due to Prof *Nils Oker-Blom* and Prof. *Antti Vaheri* for advice in this work and for critical review of the manuscript, Dr *Per Andersson* kindly performed the autopsies and offered advice in connection with histology of the sacrified chickens I am grateful to Mrs *Annikki Kelkka* for excellent technical assistance.

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PHAGOCYTOSIS OF ^{32}P LABELLED *E. COLI* BY HUMAN POLYMORPHONUCLEAR CELLS (PMN)

Adaptation of a Method

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Midtvedt, T. & Melby, K. Phagocytosis of ^{32}P-labelled *E. coli* by human polymorphonuclear cells (PMN). Adaptation of a method. Acta path. microbiol. scand. Sect. B, 86: 193–199, 1978.

A system for the study of phagocytosis by human polymorphonuclear cells (PMN) is presented. The leucocytes are harvested from heparinized whole blood by the Bøyum method and transferred to glass tubes to yield glass adherent monolayers of leucocytes, approximately 80% of which were PMN. A strain of *E. coli* labelled by ^{32}P serves as test organism.

Key words: Neutrophils, phagocytosis, *E. coli*.

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The process of phagocytosis can be divided into three main phases (18, 19), i.e. the process of recognition/adherence, the process of ingestion, and the process of killing and digestion of the ingested object by the phagocyte. Various methods have been published to study the process of phagocytosis. These can be divided into three groups: 1. Examination by microscopy of the leucocytes after incubation with the particles actually under study gives possibilities for estimating the process of ingestion (7, 13); 2. Incubation of living particles and leucocytes followed by determination of the number of living particles associated with the leucocytes and/or the number of particles left in the suspension (15), provides possibilities for evaluating the processes of ingestion and of intracellular killing of the ingested microorganisms; 3. Incubation of the leucocytes with radioactive-labelled particles provides possibilities for evaluating the processes of ingestion and of elimination (5, 6, 11, 20, 22).

The latter method has been used for the study of the influence of drugs on polymorphonuclear cells obtained from rat peritoneal cavity after casein stimulation (4, 10). The results of these studies raised the question of adaption of the method to human polymorphonuclear cells. The present report demonstrates that such adaption is possible.

MATERIALS AND METHODS

Glassware. Experiments were performed in tissue culture tubes with a 30 cm^2 flat bottom. Tubes and silicone rubber stoppers were supplied by Bellco Glass Inc., Vineland, N. J.

Cleaning of glassware and stoppers. The tissue culture tubes and stoppers were boiled in soapy water for 60 min, rinsed in running tap water for at least 2 hours,

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RESULTS

Phagocytosis. Experiments revealed that 10% serum was the optimum concentration which could be used. When the concentration of serum was above 10% no increase in phagocytosis was observed. The results of one typical experiment are shown in Fig. 1. Unless otherwise stated, 10% human serum was used in the subsequent studies.

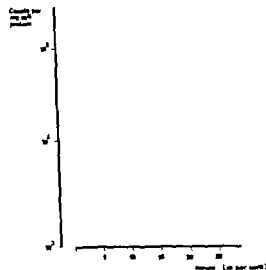


Fig. 1. Influence of serum concentration on phagocytosis. Aliquots of 2.5 ml of a logarithmic dilution of a suspension of ^{32}P-labelled *E. coli* in KRG with increasing concentrations of serum were incubated at 37° C in tubes containing monolayers of PMN. A standard incubation time of 15 min was used. Uptake of ^{32}P by the PMN was determined as outlined in »Materials and Methods«.

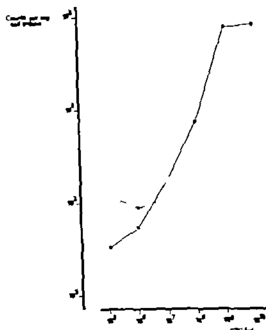


Fig. 2. Influence of number of bacteria on phagocytosis. Aliquots of 2.5 ml of a logarithmic dilution of a suspension of ^{32}P-labelled *E. coli* in KRG with 10% serum were incubated at 37° C in tubes containing monolayers of PMN. A standard incubation period of 15 min was used. Uptake of ^{32}P by the PMN was determined as outlined in »Materials and Methods«. Results from two different experiments are presented.

Experiments with various concentrations of bacteria revealed that the major increase in phagocytosis was observed when the concentration varied between 10^6 and 10^9 bacteria per ml. Consequently 10^9 bacteria per ml was chosen as the standard concentration of bacteria. (Typical experiments are shown in Fig. 2)

The influence of different times of incubation was studied, and representative experiments are shown in Fig. 3. Using an incubation period of 15 min, the ingestion was usually eight to ten times higher with serum present during the phagocytosis than with either KRG or heat-inactivated serum.

Release of label to the medium from the labelled

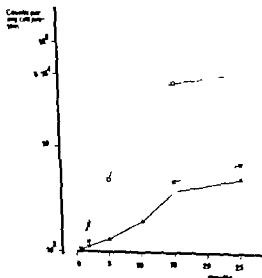


Fig. 3. Phagocytosis of ^{32}P-labelled *E. coli* suspended in various media. Aliquots of 2.5 ml of *E. coli* labelled with ^{32}P 10^9 CFU/ml suspended in three different media, (1) KRG, (2) with 10% of serum and (3) KRG with 10% heat-inactivated serum, were incubated at 37° C for various periods of time determined as described in »Materials and Methods«. □ serum, ■ heat inactivated serum, △ KRG

rinsed in 10 changes of glass-distilled water and dried in hot air

Sterilization. Glassware was sterilized with dry heat at 160° C for 90 min. Stoppers were sterilized in an autoclave at 121 C for 30 min.

Polymorphonuclear cells (PMN). Leucocytes were harvested from heparinized whole blood (5 I. U./ml) obtained by venipuncture of healthy blood donors. The maximum interval between collection of blood and beginning of the separation procedure was one hour. The leucocytes were separated as described by *Bøyum* (3), i.e. the blood was layered carefully on top of an equal volume of a solution of two parts dextran T 500 (6% in aq dest.) from Pharmacia, Stockholm, and one part Isopaque® (33.9% in distilled water) from Nyegaard & Co. Oslo. The blood was left for sedimentation at 37° C until the red cells had settled at the bottom of the tube. The leucocyte-rich layer above was then removed carefully with a pipette and washed three times with chilled Krebs Ringer phosphate buffer enriched with 10 mM glucose (KRG). Centrifugation was performed in an International centrifuge running at 200 × g at 4° C for 10 min. The concentration of leucocytes was determined in a Burker cell chamber and adjusted to $5\text{–}8 \times 10^6$ leucocytes per ml.

Aliquots of 2.5 ml of this solution were transferred to tubes with or without coverslips and incubated at 37 C. One hour later the medium was decanted and the cells washed once with prewarmed KRG (37° C) by tilting the tubes 10 times.

Differential counts revealed that the average number of PMN was 82%. The remaining cells were mononuclear cells dominated by lymphocytes.

Serum. Serum was obtained by venipuncture of several healthy blood donors with different blood groups according to the ABO system. The sera were pooled and stored in small aliquots at −80° C until use.

Bacteria. The strain used has been described earlier as a member of the species *Escherichia coli* according to Bergey's Manual (2). The strain was stored in lyophilized state at 5 C and was also kept in subculture on lactose bromthymol blue agar plates.

Culture technique. The medium was prepared according to *Benacerraf et al.* (1). The strain was cultivated in 50 ml of medium for 18 hours. Four ml of this culture was transferred to 200 ml of medium to which was added 1 mC ^{32}P labelled orthophosphate. The bacterial growth was allowed to proceed for 200 minutes and then stopped by rapid cooling to 0° C. The number of viable bacteria, determined as colony forming units (CFU) after appropriate dilution and plating, was 10^9 (± 0.5 log). The growth of the culture was followed by spectrophotometer: the optical density was 0.35 when read at 525 nm. All incubations were made aerobically in a shaker at 37° C.

Preparation of bacterial suspension. The bacteria were harvested by centrifugation at 6000 × g for 10 minutes at 0° C in a Sorwall RC2B centrifuge. The bacterial suspension was washed three times with chilled KRG and the sediment was suspended in KRG in 1/10 of the original volume. The different suspensions were prepared from this suspension and stored at 0° C until use. The storage time never exceeded 2 hours. After these manipulations, the number of viable bacteria was still within the limits determined at the end of the growth period, i.e. $10^9 \pm 0.5$ log. The content of radioactivity in the medium was less than 1% of the total radioactivity in the suspension.

Phagocytosis experiments. Unless otherwise stated, a suspension containing 10^9 of ^{32}P-labelled E. coli per ml in KRG with and without 10% human serum was used. Aliquots of 2.5 ml of this suspension were added to tissue culture tubes containing rinsed monolayers of PMN and incubated at 37° C. The period of incubation was from the moment the tubes were placed in the incubator until they were removed. On termination of incubation the bacterial suspension was poured off and replaced by 2.5 ml chilled KRG in order to stop the phagocytosis. The cells were rinsed four times with chilled KRG and after complete drying at 37° C, 2 ml Lowry's alkaline copper solution (LACS) was added to each tube to dissolve the cells. Thereafter 0.2 ml was removed for determination of radioactivity and 0.5 ml for determination of cell protein content.

Release of label to the medium. Two types of experiments were performed.

1. Tubes containing 2.5 ml of the bacterial suspension but without PMN were incubated at 37° C for various periods of time. On termination of incubation the suspensions were poured quickly into chilled centrifuge tubes and centrifuged at 6000 × g at 0° C for 10 minutes. 0.1 ml of the clear supernatant from each tube was removed for determination of radioactivity released to the medium from the bacteria. The release was expressed in counts per ml per minute.

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RESULTS

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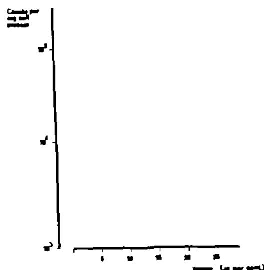


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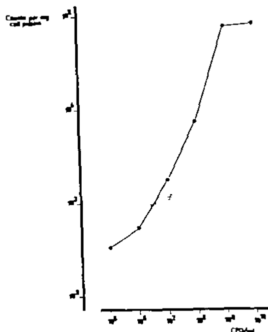


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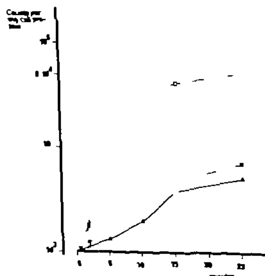


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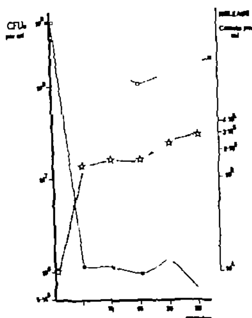


Fig. 7 Release of label from bacteria compared to the number of viable bacteria. Aliquots of 2.5 ml of a suspension of ^{32}P labelled *E. coli* in KRG with 10% serum or in KRG only were incubated at 37° C for various periods of time. Tubes in triplicate were used for each period of incubation. From one of the tubes, samples were removed immediately for determination of the number of *CFU* as expression of viable bacteria. Both tube dilution technique and colony counting were used. The two other tubes were chilled to 0°C and used determination of released ^{32}P as outlined in »Materials and Methods». ○ KRG ■ serum ⊕ release.

presented in Fig. 6. No major deviation from the results obtained without PMN could be demonstrated.

Determination of CFU. In the presence of serum, the number of CFU was reduced by 2.5 log during the first 5 min (Fig. 7). No further reduction in the number of CFU was observed until after 25 min of incubation. No marked reduction in the number of CFU was observed when only KRG was present during the incubation period.

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Aliquots of 2.5 ml of a suspension containing 10% serum and 10^{8} ^{32}P-labelled *E. coli* were incubated for 15 minutes in tissue culture tubes containing rinsed monolayers of PMN.

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The basic method of *Tripestad & Midtvedt* (20) using rat PMN has been useful in the study of different factors of host defence mechanisms (21) and the influence of drugs on these mechanisms (4, 10). The adaptation of this method to the human polymorphonuclear cells might therfore be of importance.

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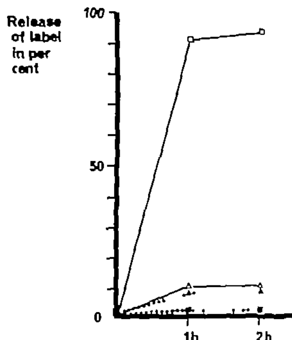


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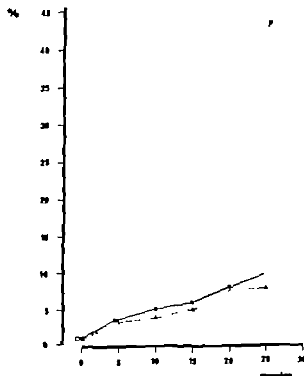


Fig 4 Release of label from bacteria incubated in various media. Aliquots of 2 5 ml of a suspension of ^{32}P labelled *E coli* 10^9/ml in three different media were incubated at 37° C for various periods of time The media consisted of (1) KRG only (2) KRG enriched with 10 % of serum and (3) KRG with 10 % of heat inactivated serum (30 min at 56 C). The total radioactivity of the bacterial suspension was 974 000 cpm per ml. The results are presented as percentage radioactivity released compared with the total radioactivity in the suspension ● KRG ■ serum, △ serum (heat inactivated).

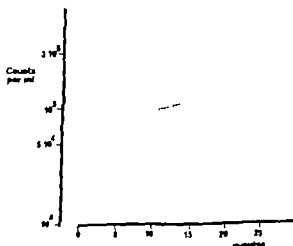


Fig 6 Release of label from *E coli* labelled with ^{32}P incubated with different media and PMN Aliquots of 2.5 ml containing 10^9 ^{32}P-labelled *E coli*/ml suspended in two different media (1) KRG with 10 % serum and (2) KRG only were incubated at 37° C in tubes with monolayers of PMN After various periods of incubation, the release of label to the suspending medium was determined as described in »Materials and Methods«. Total radioactivity in the suspension was 840 000 cpm per ml ■ serum, ▽ KRG

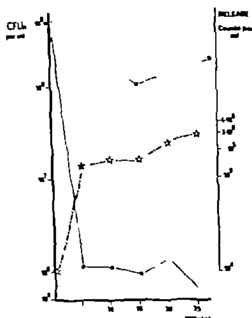


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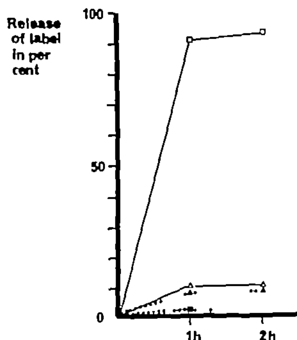


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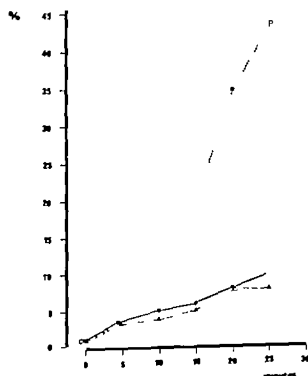


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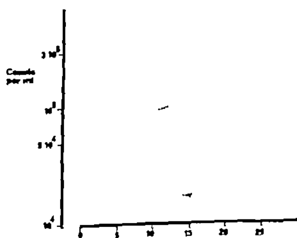


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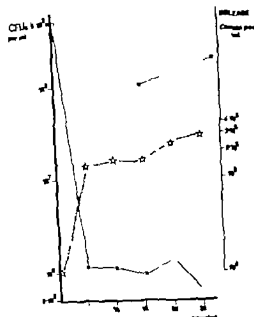


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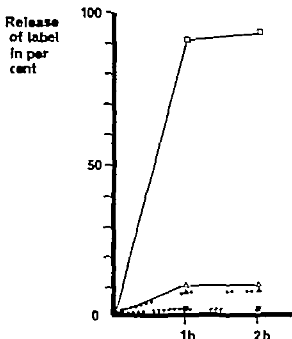


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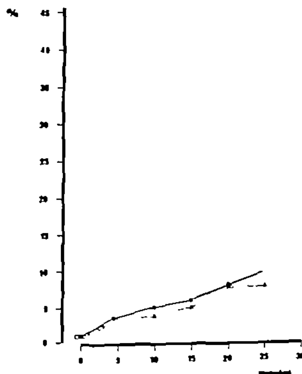


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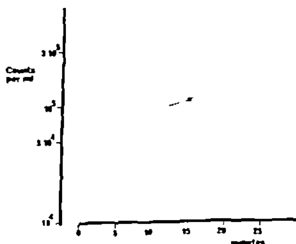


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Spitznagel (16) and *Spitznagel & Wilson* (17) have demonstrated that the effect of fresh serum on bacteria is mediated mainly by heat labile factors on the cell membranes of the bacterium. The phosphate incorporated in the cytoplasma is easily released. However a major part of the phosphate is connected to cell wall structures and other macromolecular structures that are not so easily decomposed. They have also reported a release of label from ^{32}P labelled *E. coli* of 60% when using human serum and an incubation period of 1 hour at 37° C. Much less release was observed when sera from other mamals were used.

In our experiments the release of label occurs quickly in the presence of 10% fresh serum. This might be due to bacterial cell membrane damage and leakage of phosphate to the external medium, as shown by *Spitznagel & Wilson* (17). This phosphate might be reincorporated in other bacteria, but, as has been demonstrated by *Trippestad & Midtvedt* (20) this only takes place to a very small extent. Our release studies (42% release by 25 minutes at 37° C with 10% fresh serum) are in accordance with the findings of *Spitznagel & Wilson* (17).

Trippestad & Midtvedt (20) demonstrated a 25% loss of the label after a period of incubation on 25 minutes at 37° C in the presence of 10% rat serum. As a result of our studies, an incubation period of 15 min was chosen in order to avoid an external medium enriched with the label.

The bacteria were apparently quickly blocked in their ability to form colonies on agar plates. The decrease in colony forming capacity corresponded to the release of label from the bacteria. However the expresseon of the two parameters differed. After 15 min, only 1% of the bacteria initially present were apparently able to form colonies on agar plates, whereas 10% of the label had been released. The low number of CFU does not necessarily imply the death of a corresponding number of bacteria. Besides being lethal, serum could also cause a coating of the bacteria which might result in a depressed ability to yield colonies on agar plates. It is also likely that the bacteria rendered unable to form colonies on agar plates might still be labelled by ^{32}P connected to macromolecular structures, as suggested by the studies by *Spitznagel* (16), *Spitznagel & Wilson* (17) and *Cohn* (5) on the stability of radiolabelled *E. coli*.

The bacteria were presented to PMN and the rate of ingestion seems to b in accordance with the findings of other investigators. Thus, *Forsgren* (6) using an oil emulsion as particle source for phagocytosis, reports that a period of ingestion of 10 min is sufficient. *Lehrer* (8) states that 7 min is an adequate time of exposure, and *Munoz & Geister* (12) that 15 min is a suitable period of incubation. These findings are in agreement with the results presented here and the observations published by *Truppestad & Midtvedt* (20).

By the phagocytosis of bacteria, the PMN monolayer is enriched with bacterial protein. Thus, the possibility of obtaining a false low estimate of the ingestion is present. Previous studies on rat PMN have revealed that this factor could contribute to a maximum error of 7% in the protein determinations (20).

During the experimental series, large pools of serum have been used in order to keep the serum factors as constant as possible. When using the system in future experiments, it must be taken into consideration that sera may differ in their ability to influence the process of phagocytosis. Consequently comparison of effects should only be carried out within one experiment.

The aim of this investigation was to adapt a system which is functional on rat polymorphonuclear cells to human polymorphonuclear cells and it would appear that this adaption is possible. Thus, a system is available that can be used for the study of host defence mechanisms, both humoral and cellular where the human PMN are involved. The influence of drugs on these factors may also be subjected to further study. Another important aspect of the experimental system is the fate of the ingested labelled particle.

This study was supported by grants from Norges *Almenvitenskabelige Forskningsråd*

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ASSESSMENT OF WOUND CONTAMINATION BY WOUND IRRIGATION

Experimental Investigations on Quantitative Recovery of Anaerobic and Aerobic Bacteria

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SVEND LINDENBERG

Statens Seruminstitut, Dept. of Clinical Microbiology and Surgical Dept. K, Frederiksberg Hospital, and Surgical Dept. C, Rigshospitalet, University of Copenhagen, Denmark

Scheibel, J. H., Lykkegaard Nielsen, M. & Lindenberg, S. Assessment of wound contamination by wound irrigation. Experimental investigations on quantitative recovery of anaerobic and aerobic bacteria. Acta path. microbiol. scand. Sect. B, 86: 201–05, 1978.

The quantitative recovery of E. coli, S. faecalis and B. fragilis from operative abdominal wounds was investigated in pigs in an experimental model suitable for statistical calculations. Wounds were contaminated in groups of ten with different numbers of either a single bacterial species or a mixture of two species. The wound was irrigated with saline 20 minutes after contamination. Significant differences in recovery were found between the bacterial species investigated. Expressed as percentage of the number of bacteria used for contamination, the recovery for a given species was rather low, but it was constant and independent of the degree of contamination. The investigation did not suggest any principle difference in the recovery of anaerobic and aerobic bacteria. The clinical applicability of the method is not yet clarified.

Key words: Wound contamination, wound irrigation, bacteriological sampling.

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The frequency of post-operative wound infection has not been significantly reduced, despite considerable success in decreasing the exogenous sources of bacteria. The main origin of bacteria found in post-operative incisional abscesses is endogenous, i.e. bacteria from the organs operated on, for example the gastro-intestinal or the biliary tract. To combat post-operative wound infection, it is desirable to be able to evaluate to what degree different surgical procedures contribute to wound contamination, and to what degree different devices can protect the wound from contamination.

Several methods (irrigation/rinse techniques or contact methods in different modifications) have been used to determine the number of bacteria left in the wound at closure. The quantitative bacterial recovery in these methods has been evaluated *only* as bacterial transfer from *artificial* surfaces (1, 6). It seems quite hazardous to draw conclusions as to the complicated structure of a wound surface, from artificial surfaces like an agar surface (6) or china salad dishes (1).

The wound irrigation method is easy to perform and involves the total surface of the wound. The aim of the present study was to determine the quantitative bacterial recovery from experimental wounds contaminated with known numbers of bacteria, to investigate whether different bacterial species showed variations in recovery, and to investigate whether the method was suitable for the recovery of anaerobic bacteria.

MATERIAL AND METHODS

Experimental animals, anaesthesia, surgical procedures and preparation of wounds. Pigs of the Danish landrace weighing 40 kg were used in all experiments. Anaesthesia was performed with halothane after tracheal intuba-

tion. After shaving the entire abdomen, the skin was disinfected three times with 2.5 per cent iodine in 70 per cent alcohol. Draping was made with sterile drapes and Steridrape®. Ten to twelve wounds, all 10 cm in length, were made as follows, the skin, the subcutaneous layer and the fascia covering the abdominal muscles were cut, and finally the fascia was closed with interrupted silk sutures. The wounds prepared in this investigation thus imitated a stage of closure of the abdominal wall in human laparotomies (i e. the stage just before closing the subcutaneous layer and the skin) apart from the fact that the abdominal muscles had not been divided and the peritoneum had not been opened.

Contamination of wounds In all but one experiment, contamination of each wound was made with 0 5 ml of the bacterial suspension in question. This volume was spread evenly over the sides and the base of the wound with a tiny syringe. The wound was left untouched for 20 minutes before irrigation was performed.

In one experiment, bacterial recovery from diffusely contaminated wounds was compared with bacterial recovery from wounds with localized contamination (cf Table 2). In this experiment, 10 wounds were allocated at random for diffuse or localized contamination, and the diffuse contamination was performed with 0 5 ml bacterial suspension as described above. The localized contamination was performed by depositing the same number of bacteria in a volume of 0 05 ml at one point in the wound. After 20 minutes, irrigation was carried out without knowledge of which wounds were contaminated in the diffuse or the localized manner

Irrigation of wounds. In all but one experiment, the wounds were irrigated with 5 ml saline for 30 seconds using a sterile plastic spatula, and efforts were made to irrigate all wounds in the same manner After irrigation, the saline was aspirated in a syringe and immediately examined bacteriologically as described below In one experiment, 12 wounds were allocated at random for either 30 or 60 seconds of irrigation (cf Table 1).

Test organisms. E coli (02.H4), *B fragilis* (ss. *fragilis*) and *S faecalis* all isolated from clinical specimens.

Media E coli was grown on an agar medium containing Orthana peptone 1% Oxoid yeast extract 0 5% NaCl 0 5% sodium thiosulphate 0 1% bromthymol blue 0 01% glucose 0 04% lactose 0 9% and Maranil® 0 005% Maranil® is a detergent which prevents growth of gram positive organisms. *B fragilis* was grown on prereduced nutrient agar medium containing 10% defibrinated horse blood with an admixture of 400 µg per ml kanamycin which completely prevented growth of *E coli. S faecalis* was grown on nutrient agar containing 10% defibrinated horse blood and 0 04% potassium tellurite which prevented growth of *E coli*

Pilot studies were performed to ensure that the selective media did not repress growth of the bacterial species in question to any degree. Except for the kanamycin content, the media were standard media manufactured by Statens Seruminstitut, Copenhagen, and used in routine bacteriological work. The media were dispensed in plastic petri dishes with a diameter of 9 cm.

Bacterial inocula used for contamination For each experiment, broth was inoculated from appropriate stock culture and incubated at 35 C for 18 h. In the case of *B fragilis*, prereduced broth was used and incubation performed in a Gas-pak® jar

The bacterial suspensions used for inoculating the wounds were produced by diluting the fully-grown culture in broth. At the same time as the wounds were contaminated, the concentration of the suspension in use was determined by floating the relevant agar plates with one-half ml of the suspension and one-half ml of ten-fold dilutions Four plates were used for each dilution The plates were incubated at 35 C for 24 h For *B fragilis*, incubation was performed in Gas-pak ® jars for 48 h The aim was to obtain plates containing 50–500 colonies per counting.

Irrigation fluid (Rinse). Within five minutes after irrigating a wound one-half ml of the fluid and one-half ml of ten-fold dilutions in broth were floated in duplicate onto the relevant agar plates Incubation and counting were performed as described above (cf bacterial inocula used for contamination)

Statistical calculations Non-parametrical statistical methods were used, all comparisons being made by means of Wilcoxon's test

TABLE 1 *Influence of Different Periods of Irrigation on Bacterial Recovery from Subcutaneous Wounds Contaminated With* E. coli *Six Wounds Were Examined in Each of the Two Groups All Wounds Were Irrigated with 5 ml Saline*

Number of bacteria inoculated	Period of irrigation (seconds)	Bacterial recovery after irrigation	
		Bacteria per 5 ml irrigation fluid (median and range)	Percentage of inoculated no of bacteria (median and range)
6×10^3	30	90 (40–160)	1 5 (0 6–2 7)
6×10^3	60	70 (25–270)	1 2 (0 4–4 5)

TABLE 2. *Bacterial Recovery from Subcutaneous Wounds Contaminated in Two Different Ways with the Same Number of* E coli. *Five Wounds Were Examined in Each of the Two Groups and All Wounds Were Irrigated with 5 ml Saline for 30 Seconds*

Mode of contaminating the wound	No of bacteria inoculated	Bacterial recovery after irrigation	
		Bacteria per 5 ml irrigation fluid (median and range)	Percentage of inoculated no of bacteria (median and range)
0.5 ml bacterial suspension distributed evenly throughout the wound	5×10^5	1.2×10^4 (2.6×10^3–1.3×10^4)	2.3 (0.5–2.6)
0.05 ml bacterial suspension deposited at one point of the wound	5×10^5	9.1×10^3 (4.1×10^3–1.6×10^4)	1.8 (0.8–3.2)

RESULTS

Bacterial recovery after different periods of irrigation can be seen in Table 1. No significant difference was found between wounds rinsed for 30 and 60 seconds ($p > 0.10$). It will be seen from Table 2 that similar bacterial recoveries were obtained from diffusely contaminated wounds and from wounds with focal contamination ($p > 0.10$).

TABLE 3. *Bacterial Recovery from Subcutaneous Wounds after Contamination of the Wounds with Different Doses of* E. coli *and Irrigation for 30 Seconds with 5 ml Saline. Ten Wounds Were Examined for Each Dose of* E. coli

Number of bacteria inoculated	Bacterial recovery after irrigation: Bacteria per 5 ml irrigation fluid (median and range)	Bacterial recovery after irrigation: Percentage of inoculated no. of bacteria (median and range)
0	0 (0–0)	0 (0–0)
2.0×10^1	0 (0–0)	0 (0–0)
8.8×10^1	0 (0–0)	0 (0–0)
9.0×10^2	17 (5–35)	1.9 (0.6–3.9)
7.0×10^3	93 (10–520)	1.3 (0.1–7.4)
4.0×10^5	3.8×10^3 (2.0×10^3–1.3×10^4)	0.6 (0.3–2.2)
5.0×10^7	5.0×10^5 (2.0×10^5–2.3×10^6)	1.0 (0.4–4.6

Table 3 shows the bacterial recovery after irrigation of wounds contaminated with an increasing number of *E. coli*. When less than 10^2 bacteria were used for contamination, bacteria were never recovered from the rinsing fluid. In the case of wounds contaminated with 9×10^2 to 5×10^7 bacteria, the median percentage of inoculated bacteria recovered was surprisingly constant, varying between 0.6 and 1.9. However, within each set of experiments including 10 wounds contaminated with the same number of bacteria, a considerable variation in bacterial recovery was found, the difference in percentage recovery being as high as 70-fold in one of the experiments.

Bacterial recovery from wounds contaminated with increasing numbers of *B fragilis* can be seen in

TABLE 4. *Bacterial Recovery from Subcutaneous Wounds after Contamination of the Wounds with Different Doses of* B. fragilis *and Irrigation for 30 Seconds with 5 ml Saline. Ten Wounds Were Examined for Each Dose of* B. fragilis

Number of bacteria inoculated	Bacterial recovery after irrigation: Bacteria per 5 ml irrigation fluid (median and range)	Bacterial recovery after irrigation: Percentage of inoculated no. of bacteria (median and range)
1.1×10^3	130 (20–305)	11.8 (1.8–27.7)
1.2×10^4	1.6×10^3 (8.0×10^2–3.6×10^3)	13.3 (6.7–30.0)
1.2×10^6	1.5×10^5 (0.7×10^5–3.6×10^5)	12.5 (5.8–30.0)

TABLE 5 *Bacterial Recovery from Subcutaneous Wounds after Contamination with Different Doses of* E. coli *or* E. coli + B fragilis *and Irrigation of the Wounds for 30 Seconds with 5 ml Saline Six Wounds Were Examined for Each Dose of* E. coli *or* E. coli + B fragilis

Number of bacteria inoculated		Bacterial recovery after irrigation as percentage of inoculated number (median and range)	
E coli	*B fragilis*	*E coli*	*B fragilis*
3.0×10^3	7.5×10^3	4 4 (1 9–6 9)	24 4 (6.3–32 0)
3.0×10^3		5 5 (4 0–7 4)	
3.0×10^5	5.4×10^5	6 6 (4 5–8 9)	33 1 (26 6–45 7)
3.0×10^5		3 0 (1 2–5 2)	

Table 4 The median percentage of the number of inoculated bacteria recovered from the rinsing fluid was constant at about 12 per cent in all three sets of experiments, independent of the degree of contamination investigated. As in the experiments with *E coli* (Table 3), this constant recovery expressed as a median percentage of the inoculum concealed a considerable variation within each set of experiments including 10 wounds.

TABLE 6 *Bacterial Recovery from Subcutaneous Wounds after Contamination with Different Doses of* E. coli + S faecalis *and Irrigation of the Wounds for 30 Seconds with 5 ml Saline Ten Wounds Were Examined for Each Dose of* E coli + S faecalis

Number of bacteria inoculated		Bacterial recovery after irrigation as percentage of inoculated number (median and range)	
E coli	*S faecalis*	*E coli*	*S faecalis*
3.4×10^3	2.7×10^3	4 6 (1 8–8 2)	30 9 (8 1–55 4)
3.2×10^5	2.7×10^5	5 1 (1 0–7 1)	37 3 (15 1–63 0)

To elucidate whether the difference in bacterial recovery of *E coli* and *B fragilis* (Tables 3 and 4) was real, or a result of day-to-day and animal-to-animal variation, the experiment outlined in Table 5 was carried out. It will be seen that a significantly better recovery was obtained with *B fragilis* than with *E coli* both when the wounds were contaminated with 10^3 and with 10^5 bacteria ($p < 0.001$ for both comparisons).

Table 6 shows that *E coli* and *S faecalis* were recovered at different rates after rinsing, both when the wounds were contaminated with 10^3 and with 10^5 bacteria ($p < 0.001$ for both comparisons)

DISCUSSION

The present investigation revealed that bacterial recovery from subcutaneous wounds after irrigation was surprisingly low and that there were significant differences in bacterial recovery between different bacterial species. The structure of the wounds (i.e. the abdominal muscles were not divided and the peritoneum was not opened (cf methods)) prevented any loss of inoculated bacteria or rinsing fluid from the wounds. Bacterial adhesion to the wound surface (i.e. the subcutaneous layer and the fascia including fascial sutures in the base of the wound) is a likely explanation for the rather low bacterial recovery Bacterial adhesion to the wound surface presumably depends on the surface properties of the bacterial species in question. Different surface properties might explain the significant difference in bacterial recovery between *E coli*, *B fragilis* and *S faecalis*. It was remarkable, and to some degree unexplainable, that the bacterial recovery within each of four experiments (cf Tables 3 4 5 and 6) was rather constant for a given bacterial species when the recovery was expressed as percentage of the number of bacteria used for contamination This was true even when the number of bacteria used for contamination ranged from 9×10^2 to 5×10 (cf Table 3) If it is true that adhesion of bacteria to the wound surface is responsible for the decreased bacterial recovery this adhesion was not influenced by different periods of irrigation (cf Table 1) Factors other than adhesion could theoretically decrease the bacterial recovery In the present experimental model, only 20 minutes elapsed from contamination of the wound to time of rinsing and this short interval does not favour the conception that phagocytosis of bacteria or invasion of bacteria into deeper layers of the wound plays any major role (9) The possible importance of other antibacterial factors in the wound (i.e. from wound tissue, from wound

exudate and from minor amounts of blood in the wound) is not elucidated in the present experimental model, nor is it elucidated whether the viable count method has concealed bacterial agglutination in the rinsing fluid. Since the irrigation fluid was plated within 5 minutes, the killing effect of saline can be disregarded (10).

With the present experimental model, it was possible to demonstrate marked differences in bacterial recovery between *E. coli*, *B. fragilis* and *S. faecalis*. However, it will be seen from Tables 3, 4 and 6, that there is large variation within any single experiment involving 10 wounds (cf. median and range in the tables), despite efforts to produce uniform wounds, to contaminate the wounds in exactly the same manner and to irrigate all wounds in the same way for the same period of time. Tables 1 and 2 show that small differences in the distribution of the inoculum used for contamination or in the time of irrigation hardly contribute to the variation within a single experiment, and the bacteriological technique is hardly responsible for this variation (cf. methods).

The question as to whether the wound irrigation method is suitable for clinical use is, in our opinion, not yet settled, despite the fact that the method has been used by several investigators in different modifications for quantitative determination of wound contamination (2, 3, 4, 7, 8). The present investigation seems to indicate that bacterial contamination with less than approx. 10^2 *E. coli* remains undetected. We do not know yet whether this slower limit is true for most bacterial species, nor do we know whether or not this slower limit is crucial, since the number of bacteria necessary for establishing wound infection is still under discussion (5). The species-dependent recovery found in the present study is not easy to explain, and further investigations are necessary to elucidate whether it invalidates the method for clinical use. Experimental comparisons of different methods (i.e. irrigation methods and contact methods such as the velvet pad (6)) would be desirable in order to determine the method of choice for assessment of contamination of operation wounds.

The skilful technical assistance of *Jette Hello* is gratefully acknowledged.

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Number of bacteria inoculated		Bacterial recovery after irrigation as percentage of inoculated number (median and range)	
E. coli	*B. fragilis*	*E. coli*	*B. fragilis*
3.0×10^3	7.5×10^3	4.4 (1.9–6.9)	24.4 (6.3–32.0)
3.0×10^3		5.5 (4.0–7.4)	
3.0×10^5	5.4×10^5	6.6 (4.5–8.9)	33.1 (26.6–45.7)
3.0×10^5		3.0 (1.2–5.2)	

Table 4. The median percentage of the number of inoculated bacteria recovered from the rinsing fluid was constant at about 12 per cent in all three sets of experiments, independent of the degree of contamination investigated. As in the experiments with *E. coli* (Table 3), this constant recovery expressed as a median percentage of the inoculum concealed a considerable variation within each set of experiments including 10 wounds.

TABLE 6 *Bacterial Recovery from Subcutaneous Wounds after Contamination with Different Doses of* E. coli + S. faecalis *and Irrigation of the Wounds for 30 Seconds with 5 ml Saline. Ten Wounds Were Examined for Each Dose of* E. coli + S. faecalis

Number of bacteria inoculated		Bacterial recovery after irrigation as percentage of inoculated number (median and range)	
E. coli	*S. faecalis*	*E. coli*	*S. faecalis*
3.4×10^3	2.7×10^3	4.6 (1.8–8.2)	30.9 (8.1–55.4)
3.2×10^5	2.7×10^5	5.1 (1.0–7.1)	37.3 (15.1–63.0)

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ANALYSIS OF AMINES AND OTHER BACTERIAL PRODUCTS BY HEAD-SPACE GAS CHROMATOGRAPHY

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Larsson, L., Mårdh, P.-A. & Odham, G. Analysis of amines and other bacterial products by head-space gas chromatography. Acta path. microbiol. scand. Sect. B, 86: 207–213, 1978.

A gas chromatographic (GC) head-space technique is presented, which is suitable for the analysis of volatile products in bacterial broth cultures. This is exemplified by studies on *Clostridium septicum*, *Klebsiella pneumoniae* and *Proteus mirabilis*. The media were acidified or made alkaline and after heating, samples of the gas phase above the media were directly injected into the gas chromatograph. A gas chromatograph equipped with dual columns and flame ionization detectors was used, employing Porapak Q and Chromosorb 103 as stationary phases. Analyses of acidified media, using Porapak Q, gave chromatograms representing acidic and neutral volatile products, while when analysing samples made alkaline, using Chromosorb 103, alkaline and neutral compounds could be detected. Interest was particularly concentrated on the analysis of bacterial amines. *P. mirabilis* was found to produce isobutylamine and isopentylamine, which were identified by mass spectrometry and GC retention times. *C. septicum* produced ethylamine. The GC head-space technique described constitutes a means for rapid identification of microorganisms. It is adaptable for use on a routine basis in the clinical microbiological laboratory.

Key words: Gas chromatography; head-space analysis; bacteria.

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Gas chromatographic (GC) analysis of volatile microbial products can be used as an aid in the differential diagnosis of microorganisms. The GC techniques usually employed include extraction of liquid culture media using an organic solvent, followed by analysis of the organic phase. Direct injection of broth cultures into the gas chromatograph (2, 20), and analysis by GC of the head-space gas above the media (1, 8, 10, 12, 17, 18, 19), have also been used. Head-space GC was recently reported to be of use in the identification of anaerobes and in diagnosis of urinary tract pathogens (13, 14, 17).

In GC analysis of microorganisms, acidic and neutral products have generally been studied. Detection of amines may, however, also prove valuable for species differentiation, but has hitherto usually involved rather time-consuming derivatization procedures (3–7).

The aim of the present study was to develop a head-space GC technique allowing analysis of acidic, neutral and alkaline volatile products of microorganisms grown in broth media. The potential capacity of the technique for the rapid identification of microorganisms is discussed.

MATERIALS AND METHODS

Organisms

One freshly isolated strain of *Clostridium septicum*, two strains of *Klebsiella pneumoniae* and four strains of *Proteus mirabilis* were used.

Media and Culture Technique

C. septicum was stored in Gaspak jars at 37°C before culturing under anaerobic conditions in chopped-meat

Acta path microbiol scand Sect B 86 207-213 1978

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(CMG) and peptone-yeast extract (PYG) medium, each supplemented with 1% (w/v) of glucose. The media were prepared as recommended elsewhere (15). *K pneumoniae* and *P mirabilis* were cultured in Trypticase soy broth (TSB) (Oxoid), and in CMG (15) without cysteine hydrochloride. All strains studied were incubated in 5 ml of medium at 37°C for 24 hours before use. Each strain was cultured and analysed three times. For comparison, sterile media were also incubated under the same conditions as for the inoculated media, and further analysed

Standard Solutions

A standard solution was prepared by adding 20μl each of ethanol, *n*-propanol, *n* butanol and *n*-amyl alcohol, 0 5 ml each of acetic, propionic, *n*-butyric, *n* valeric, isocaproic and *n*-caproic acid and 0 25 ml each of isobutyric and isovaleric acid to 500 ml of distilled water Another standard solution was prepared by adding 20 μl each of the above-mentioned alcohols and of ethylamine, *n* propylamine and *n*-butylamine, and 40μl each of *n*-pentylamine and *n*-hexylamine to 500 ml of distilled water Three other solutions were prepared by adding 1 ml each of octanoic and nonanoic acid 50 μl each of octanol and decanol, and 50 μl each of decylamine and dodecylamine to 500 ml of distilled water respectively The standard chemicals used had a minimum purity of 97% (w/v) except ethylamine which was available in a 68% (w/v in water) concentration (Merck).

Preparation of Samples for Head-Space GC Analysis

Two ml of sterile medium, 2 ml inoculated medium and 2 ml of the standard solution containing only alcohols, were transferred to two 10 ml ampoules containing 1 5 g of anhydrous Na_2SO_4. To one of the ampoules were added 5 drops (approximately 0 1 ml) of diluted H_2SO_4 (1/3 v/v in water) and to the other 4 pellets of solid NaOH (analytical grade) (approximately 0 4 g) Standard solutions containing acids were acidified, and those containing amines made alkaline prior to analysis The samples were held at 75°C Two ml of the vapour above the aqueous samples were collected with a gas-tight syringe (Hamilton 1005 LT) and injected into the gas chromatograph. The sealing and heating procedures used were those described previously (17)

Gas Chromatographic Equipment and Test Conditions

A Varian gas chromatograph equipped with dual flame ionization detectors (model 2440) was used Two 2 m glass columns with internal diameters (i.d) of 2 mm one packed with Chromosorb 103 80/100 mesh (Johns-Manville, USA) and one with Porapak Q 80/100 mesh (Waters Ass USA) were employed The injector and detector temperatures were held at 245°C and the nitrogen carrier gas flow was 25 ml/min. The Chromosorb 103 column was usually operated at 170°C and the Porapak Q column at 210°C However when analysing the acids, alcohols, and amines of higher molecular weights, the temperature of both columns was raised to 240°C The latter compounds were also analysed by injection of 0.5 μl of the pure chemicals: alkaline compounds were injected into the Chromosorb 103 column and acidic on Porapak Q while the neutral compounds were analysed on both stationary phases.

Alcohols and amines were also analysed on a Hewlett-Packard, model 5750 gas chromatograph. Here a 2 m stainless steel column (i.d. 2 mm) was used, employing a stationary phase of 10% of Carbowax 20M supplemented with 2% (w/w) of KOH on Chromosorb W AW DMCS 80/100 mesh The temperature of injector and detector was 170°C the column temperature 60°C and the nitrogen carrier gas flow 30 ml/min.

The acidified samples were regularly analysed on Porapak Q and the alkaline ones on Chromosorb 103 However in some experiments, acidified samples were analysed on the Chromosorb 103 phase Compounds present in vapours above media made alkaline, but absent in vapours above the acidified samples, were considered to represent bacterial amines. These compounds were identified by mass spectrometry and by their gas chromatographic retention times.

Mass Spectrometry

The spectra were run on a Varian Mat, model 112 GC MS combination. The GC column consisted of a 2 m glass tube (i d 2 mm) packed with Chromosorb 103 80/100 mesh. The GC conditions were as mentioned above. The ion source was held at 250°C and the electron energy was 80 eV

RESULTS

Standard Solutions

Fig 1 shows chromatograms representing the head-space atmosphere above the standard solutions containing acids and alcohols, and amines and alcohols. When analysing the vapour above acidic samples on Porapak Q acids and alcohols were detected (Fig. 1A) However propionic acid and *n* butanol did not separate under the prevailing conditions. When employing Chromosorb 103 the alkaline samples gave chromatograms representing alcohols and amines (Fig. 1B) When acidic samples were analysed on Chromosorb 103 only alcohols were detected, because acids are retained on the column (Fig 1C). Analysis of the alkaline standard solution on Chromosorb 103 after an injection of vapour containing acids yielded very small amine peaks while the sizes of the alcohol peaks were unaffected by injection of acids This was presumably because most of the amines were adsorbed on the column forming salts. However larger amine peaks were found after repeated injections of amine-containing vapours

Chromatograms obtained from the analysis of the alkaline standard solution of alcohols and amines, employing KOH-supplemented Carbowax 20M as stationary phase, showed a satisfactory separation of the compounds studied (Fig 1D).

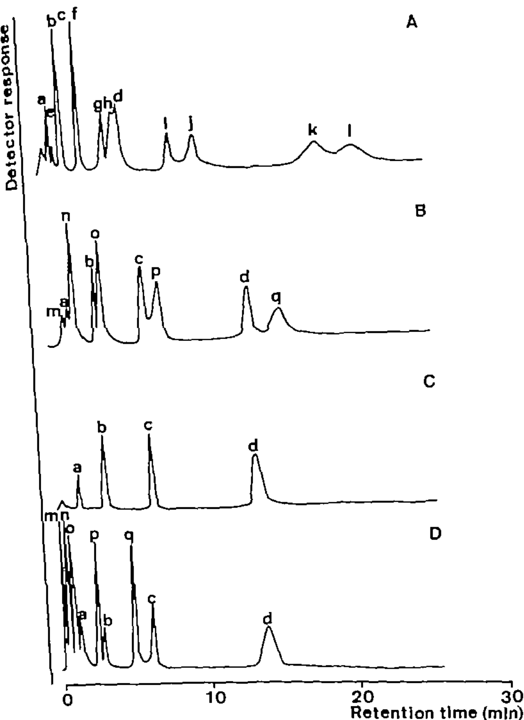


Fig. 1 Chromatograms, obtained by gas chromatographic analysis of the head-space vapour above standard solutions containing alcohols and fatty acids (A and C) and alcohols and amines (B and D), using Porapak Q (A), Chromosorb 103 (B and C) and KOH-supplemented Carbowax 20M (D) as stationary phase. Symbols: a = ethanol, b = *n*-propanol, c = *n*-butanol, d = *n*-amyl alcohol, e = acetic acid, f = propionic acid, g = isobutyric acid, h = *n*-butyric acid, i = isovaleric acid, j = *n*-valeric acid, k = isocaproic acid, l = *n*-caproic acid, m = ethylamine, n = *n*-propylamine, o = *n*-butylamine, p = *n*-pentylamine and q = *n*-hexylamine.

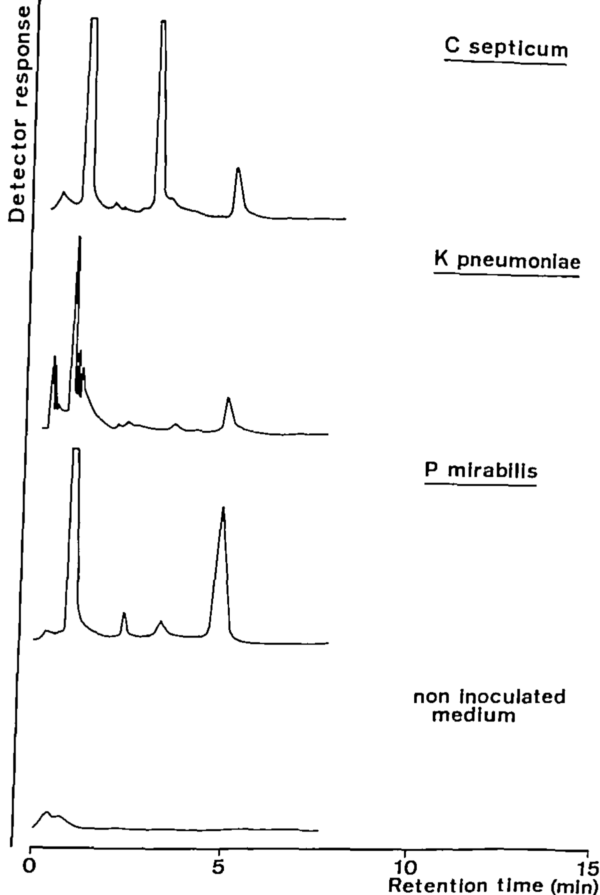


Fig 2 Chromatograms, obtained by gas chromatographic analysis of the head-space vapour above acidified chopped-meat-glucose (CMG) medium inoculated with *Clostridium septicum*, *Klebsiella pneumoniae* and *Proteus mirabilis*, using Porapak Q as stationary phase. For comparison, a chromatogram from a sample of the gas phase above sterile CMG is shown

Alcohols, Acids and Amines of Higher Molecular Weight

From the GC stationary phases used, i.e. Chromosorb 103 and Porapak Q, it was possible to elute decanol, nonanoic acid and dodecylamine at reasonably short retention times when the pure compounds were injected into the gas chromatograph at a column temperature of 240°C. When employing the head-space GC technique, octanol, octanoic acid and decylamine were the compounds of highest molecular weights that could be detected using a sample temperature of 75°C.

Analysis Using Porapak Q

Chromatograms obtained when analysing acidified CMG (pH 1.0–1.3) that had been inoculated with the three bacterial species studied, and of non-inoculated CMG are shown in Fig. 2. The atmosphere above the heated broth cultures contained appreciable amounts of volatiles which could be detected by the flame ionization detector while the non-inoculated medium gave rise only to very small peaks. The chromatographic elution profiles for the three organisms differed considerably (Fig. 2). Also chromatograms obtained from different analyses of

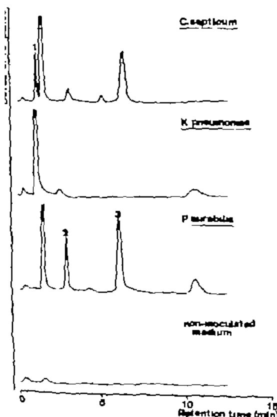


Fig. 3. Chromatograms, obtained by gas chromatographic analysis of the head-space vapour above chopped-meat-glucose (CMG) medium made alkaline and inoculated with *Clostridium septicum*, *Klebsiella pneumoniae* and *Proteus mirabilis*, using Chromosorb 103 as stationary phase. For comparison, a chromatogram representing sterile CMG is also shown. Peak 1 in the chromatogram of *C. septicum* represents ethylamine, while peak 2 in the *P. mirabilis* chromatogram represents isobutylamine and peak 3 isopentylamine.

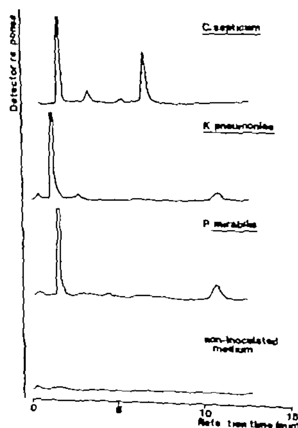


Fig. 4. Chromatograms, obtained by gas chromatographic analysis of the head-space vapour above acidified chopped-meat-glucose (CMG) medium inoculated with *Clostridium septicum*, *Klebsiella pneumoniae* and *Proteus mirabilis*, using Chromosorb 103 as stationary phase. For comparison, a chromatogram representing sterile CMG is also shown.

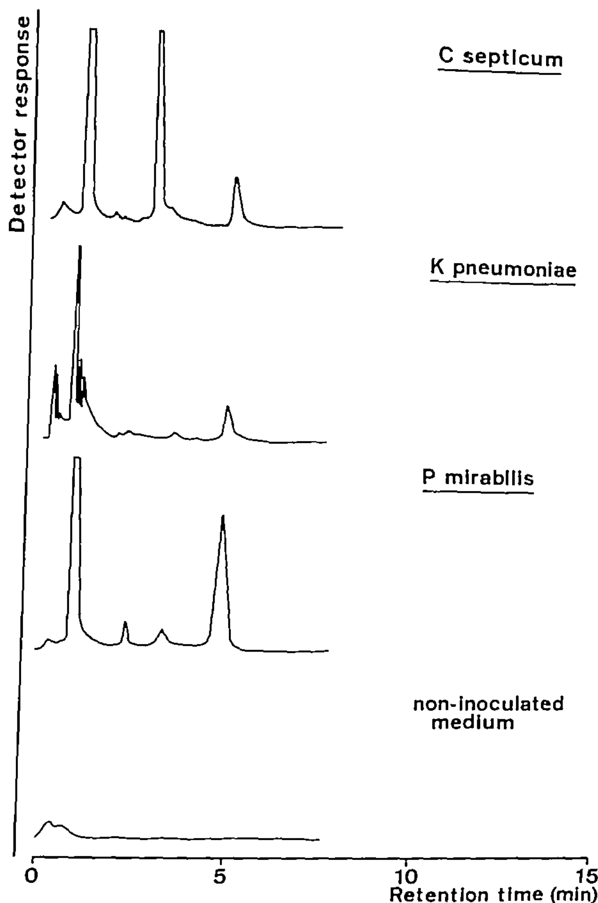


Fig 2 Chromatograms obtained by gas chromatographic analysis of the head-space vapour above acidified chopped meat-glucose (CMG) medium inoculated with *Clostridium septicum*, *Klebsiella pneumoniae* and *Proteus mirabilis*, using Porapak Q as stationary phase. For comparison, a chromatogram from a sample of the gas phase above sterile CMG is shown

Head-space GC may be automated (16). After incubation, media can be transferred to ampoules containing Na_2SO_4 and H_2SO_4 or NaOH which are ready for use in a head-space analyzer. Acidic samples are analysed using Porapak Q as stationary phase, while the alkaline samples are chromatographed on Chromosorb 103. Provided a gas chromatograph equipped with dual detectors is used, one and the same instrument can be used in all analyses.

This study was supported by the *Swedish Association Against Heart and Chest Diseases*, by grant No. 16X-4509 from the *Swedish Medical Research Council* and by a grant from the *Swedish National Science Research Council*.

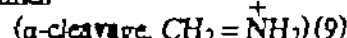
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the different strains tested of the same species were virtually identical.

Analysis Using Chromosorb 103

In Fig. 3 are shown chromatograms representing the head-space atmosphere above CMG medium inoculated with the three bacterial species studied and of sterile CMG after the media had been made alkaline (pH 12 3–12 6) and heated. Characteristic and reproducible elution patterns were obtained for each of the bacterial species studied. When injecting acidified samples of *C septicum* and *P mirabilis* on the Chromosorb 103 column, peak 1 (produced by *C septicum*) and peaks 2 and 3 (produced by *P mirabilis*) were no longer present in the chromatograms (Fig 4) These peaks were therefore considered to represent amines. The retention time of authentic ethylamine agreed with that of peak 1 which also eluted more slowly than methylamine, though faster than iso-propylamine. The mass spectra of components 2 and 3 indicated molecular weights of 73 and 87 respectively Both spectra showed base peaks at m/e 30 consistent with spectra of primary amines


(α-cleavage, $CH_2 = \overset{+}{N}H_2$)(9)

However it is difficult to distinguish between straight-chain and iso-branched isomers on the basis of mass spectra alone. Therefore, the identities of components 2 and 3 were established by GC. It was found that the retention times of peak 2 and 3 coincided with those of isobutyl and isopentylamine, respectively In addition the straight-chained amines showed considerably longer elution times.

Influence of Culture Media

C septicum produced larger amounts of ethylamine when cultured on CMG than when PYG was used. When testing *K pneumoniae* or *P mirabilis*, no conclusive differences between the chromatograms were found when using CMG or TSB.

When testing sterile CMG PYG and TSB which had been incubated under the same conditions as the bacterial cultures studied, negligible peaks were obtained compared with those found when studying cultures of the three organisms grown in the media mentioned

DISCUSSION

GC analysis of microbial metabolites in liquid culture media has proved a useful aid in the identification of microorganisms, particularly of anaerobic bacteria. Usually alcohols and fatty acids have been studied However also carbohydrates, esters, amines and sulphur-containing microbial products have been analysed. Alkaline products of bacteria belonging to the genera *Clostridium, Neisseria Proteus, Staphylococcus* and *Streptococcus* have been studied (3–7) The analytical technique has involved acylation of amino groups with heptafluorobutyric or trifluoroacetic anhydride. The rather complex procedures involving extraction plus the above-mentioned derivatizations, make these techniques less suitable for routine use in identification of clinical isolates of bacteria. However our technique, employing Chromosorb 103 allowed direct analysis of amines without derivatization. GC analysis of amines has earlier proved useful in the differential diagnosis of microorganisms (4 6 7).

The head-space GC method described is a rapid and easily performed way of analysing acidic, neutral and alkaline volatile products formed in bacterial broth cultures. Under the test conditions used, straight-chained fatty acids and alcohols up to C8 and amines up to C10 could be detected. As an aid in peak identification, acidified samples can be injected on the Chromosorb 103 column, where acids are retained.

Decrease in peak sizes, or disappearance of peaks in chromatograms found when injecting amines on a Chromosorb 103 phase containing some adsorbed free acids, indicates the formation of salt on the stationary phase. It is likely that large amounts of salt or amides possibly formed will gradually deteriorate the column, making it necessary to replace the first part of the packing material after the column has been in use for some time. It is also advisable to inject a standard solution of amines after each injection of acids on Chromosorb 103 in order to neutralize free acids adsorbed on the packing material.

In the present study Chromosorb 103 was chosen as column packing when analysing amines and alcohols. Carbowax 20M supplemented with KOH provided a satisfactory separation between these compounds (Fig. 1). However this phase is unstable at the working temperature used for the Porapak Q column, which is simultaneously present in the instrument. For this reason Chromosorb 103 was preferred to Carbowax 20M

Head-space GC can be used for analysing compounds which, at a convenient working temperature, have a sufficiently high vapour pressure (11). Since no solvent peaks are present in head space chromatograms, compounds with very short retention times can be detected. Our head-space technique was found to provide a potential tool for the characterization of both aerobic and anaerobic bacteria. Our results particularly indicate the usefulness of analysis of amines in the differential diagnosis of bacteria.

Head-space GC may be automated (16). After incubation, media can be transferred to ampoules containing Na_2SO_4 and H_2SO_4 or NaOH, which are ready for use in a head-space analyzer. Acidic samples are analysed using Porapak Q as stationary phase, while the alkaline samples are chromatographed on Chromosorb 103. Provided a gas chromatograph equipped with dual detectors is used, one and the same instrument can be used in all analyses.

This study was supported by the *Swedish Association Against Heart and Chest Diseases*, by grant No 16X-4509 from the *Swedish Medical Research Council* and by a grant from the *Swedish National Science Research Council*.

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EXPERIMENTAL ENDOCARDITIS IN RABBITS

I. Technique and Spontaneous Course of Non-bacterial Thrombotic Endocarditis

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Gutschik, E. & Christensen, N. Experimental endocarditis in rabbits. I. Technique and spontaneous course of non-bacterial thrombotic endocarditis. Acta path. microbiol. scand. Sect. B, 86: 215–221, 1978.

Insertion of a polyethylene catheter into the heart was used for regular establishment of sterile endocarditis in 52 rabbits. The catheter was inserted with the aid of guide wire, and distance marking on the catheter was used to obtain precise positioning, so that the catheter was retained with the curved distal end inside the left ventricle of the heart. The results showed that the catheterization procedure could be carried out with a high degree of accuracy. Uniform localization of the vegetations was obtained, these reaching a suitable size of development in all rabbits after insertion of the catheter for 3 days. Histological examination of the sterile vegetation showed that catheter withdrawal at this time would still permit the regular development of an infection. After withdrawal of the catheter, the sterile vegetations rapidly decreased in size and disappeared almost completely by 10 days. Sterile endocarditis in rabbits induced by a catheter for a period of 3 days proved to be a harmless and self-limiting disease. The model presented seems to be suitable for further investigations on experimental bacterial endocarditis.

Key words: Experimental endocarditis, non-bacterial thrombotic endocarditis, rabbits, polyethylene catheter.

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Of the many methods tried within the last 100 years with the aim of producing in animals an experimental model of human endocarditis, the most successful was described in 1970 by *Garrison & Freedman* (6) and further improved on by *Durack et al.* in 1972–73 (1, 2). Their method is based on the insertion of a polyethylene catheter into the interior of the heart, resulting in a non-bacterial thrombotic endocarditis, and subsequent injection of bacteria into the blood stream.

As it would seem possible with this model to perform therapeutic trials regarding the effect of various antibiotic combinations on bacterial endocarditis, a series of experiments was started with such trials as the ultimate goal (3, 4, 5, 7, 8, 11, 12).

This first paper is a report on the technique finally adopted. A description is given of certain improvements which make it possible to produce, with absolute certainty, endocarditis characterized by great uniformity of the pathological process, even if the catheter is removed before injection of bacteria. Data are presented concerning the spontaneous course and character of the pathological process of non-bacterial endocarditis. These data were obtained by performing autopsy on rabbits killed at regular intervals after insertion of the catheter.

Group III 10 rabbits were used for long-term study of sterile endocarditis. The average age of these rabbits was 190.5 days (range 171–208 days) and the average weight 2950 g (range 2790–3080 g). The catheter was removed after insertion for 3 days, after which the rabbits were observed for 60 days and then sacrificed.

RESULTS

Localization of Inserted Catheters

The curved end of the catheter was found regularly near the apex inside the left ventricle in all rabbits (group I, Fig. 1A and 1B). No unintentional injury of aorta, the leaflets or the myocardium was observed, nor was hemopericardium observed in any case. In rabbits examined after removal of the catheter certain indications of previous catheterization were found, such as vegetations or whitish, fibrous changes of the myocardium in the left ventricle.

Pathology of the Heart

Predominance, gross appearance and weight of vegetations. Uniform localization and size of the vegetations were found in rabbits which were sacrificed at the same time. The majority of the vegetations were in the apex of the left ventricle, on the base of the papillary muscles, 2–5 mm above the valves in the aorta, and on the aortic valves – usually on one or two of them, but hardly ever on all three. Furthermore, vegetations were also observed frequently around the orifice of a. anonyma. Whereas the vegetations were rather flat in the apex and on the papillary muscles, they were polypous and aceniform on the valves and in the aorta, varying in size from 1–5 mm. The vegetations were whitish and rather firm, but did not adhere to the catheter. Small vegetations could be seen with the naked eye in one of three rabbits 12 h after insertion of the catheter and in all rabbits after 24 h (group I). Vegetations had developed to an experimentally desirable size and at the typical sites mentioned above after insertion of the catheter for 3 days. Vegetations were never observed on the tricuspid valves in the left side of the heart. While the vegetations continued to increase in size and weight in rabbits with indwelling catheters (Table 1), they decreased in size and weight after withdrawal of the catheter (Table 2). Only small vegetations

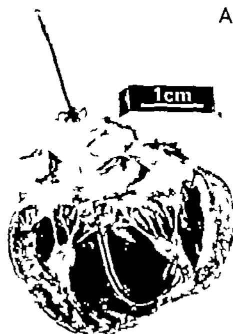


Fig. 1A. Position of the polyethylene catheter in the left side of the heart of the rabbit.

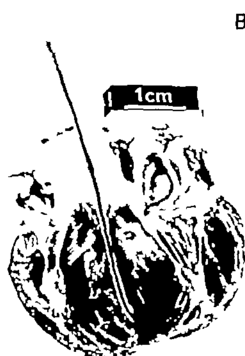


Fig. 1B. Position of the polyethylene catheter in the left side of the heart of the rabbit. The atrioventricular valves and the aorta are cut through.

MATERIALS AND METHODS

Rabbits All animals were random-bred conventional, male albino rabbits (Ssc:CPH) from Statens Seruminstitut, weighing about 3000 g. They were fed with a pellet diet supplemented with unlimited water and kept in solid cages.

Catheter The catheter was a transparent polyethylene tubing PE 90 with curved end (Surgimed Ølstykke, Denmark) Inside diameter was 0 85 mm, outside diameter 1 3 mm and length 20 cm. The catheter was marked at 9 and 11 cm from the tip It was flanged at the proximal end and fitted with a nylon female luer lock.

Guide wire Designation A 90 length 30 cm, composed of a spirally wired tube and a straight movable core (Surgimed). The diameter was 0 71 mm equal to 0 028 inches, corresponding to US No 28

Operation technique The rabbits were anaesthetized i v with mebumal natrium. The average dose at the first operation was 21 7 mg/kg, SD 3 2 (49 observations), and at the second operation 21 3mg/kg SD 2 7 (30 observations). The operation field was shaved, disinfected with spiritus jodi (Ph. Dan. 1948) and infiltrated with 3 ml of 1% lidocain chloride. Using sterile technique throughout, the right carotid artery was exposed at the midpoint of the neck, held between two silk threds, ligated proximally and dripped with 0.2 ml of 1% pethidin chloride in order to obtain a greater diameter of the artery by relaxation.

Catheterization technique In preparation for insertion of the catheter the catheter-guide wire complex was assembled as follows. First, the guide wire was inserted into the catheter and pushed forward until it was about 2 cm beyond the straightened distal end, secondly a brief retraction of the core of the guide wire was made, resulting in 2 cm of pliable tubing protruding from the distal end of the catheter

Through an incision the catheter and guide wire were passed down the carotid artery up to the 9 cm marking, then the guide wire was removed and the insertion completed by pushing the catheter up to the 11 cm marking. Preliminary investigations, which included measurement of blood pressure at different levels inside the heart, X ray and post mortem examination, showed that the tip of the catheter was at the level of the aortic valves after about 9 cm, and near the apex cordis after insertion of about 11 cm After complete insertion, the catheter was tied to the carotid artery and blood samples could now be taken through the catheter during the operation Any part of the catheter which protruded was then cut off the cervical end clamped off and the skin closed with silk sutures Removal of the catheter could be performed by simple withdrawal after new anaesthetization with mebumal natrium, but without local infiltration with lidocain chloride. Insertion of the catheter took 20–30 min, and the removal 10–20 min.

The operative mortality which was $< 10\%$ was due to bleeding from the operation wound and pneumonia. Cardiac arrhythmia was suspected in two rabbits which succumbed immediately after insertion of the catheter Symptoms and death occurred within the first 3 days of catheterization. No deaths were observed in direct connection with the anaesthetic agent.

Blood cultures. 1 ml blood was drawn from the marginal ear vein into a syringe previously soaked with 0 9% saline containing 10 U Heparin® (Leo Laboratories, Copenhagen) per ml and was spread immediately on to three 5% blood agar plates (diameter 13 5 cm). The plates were incubated at 35°C and observed for growth after 48 h Blood samples were taken from all rabbits at least twice weekly and terminally

Post mortem Examination

The rabbits were sacrificed at the predetermined times, by rapid injection of 200 mg mebumal natrium, and autopsy took place immediately The heart and spleen of all rabbits were weighed and liquid from the pleural spaces and the peritoneal cavity was collected and measured The vegetations from the left side of the heart were removed aseptically weighed and homogenized in glass tissue grinders, after the addition of 10 times saline per unit of weight. To determine the efficacy of homogenization, gram-stained slides were prepared from each suspension. Culture was made from the undiluted suspension by spreading triplicates of 0 1 ml samples on blood agar plates which were incubated for 24 h at 35°C and observed for growth.

Routine bacteriological culture was made from the peritoneal cavity liver spleen, kidneys, mesenteric glands, pleural spaces and lungs by inoculating blood agar plates with small pieces of tissue or fluid taken with a small platinum loop The plates were incubated for 48 h at 35°C and observed for growth.

The heart of one rabbit in each experimental series was used exclusively for histological examination Sections were prepared from the aorta at the level of the orifice of a. anonyma, above the leaflets, from the aorta ostium, from underneath the ostium, from the septum and papillary muscles and from the myocardium at the bottom of the left ventricle

The gross structure of the liver spleen, mesenteric glands, kidneys and suprarenal glands was examined, and where pathological processes were observed or suspected, histological examination was also carried out

Plan of the Study

Experimental Animals

Group I 21 rabbits were studied for sterile endocarditis with indwelling catheters. The average age of the rabbits was 185 1 days (range 171–223 days) and the average weight 3063 g (range 2850–3250 g) Three rabbits were sacrificed at 3 and 12 h and at 1 2 3 4 and 5 days after insertion of the catheter

Group II In 21 rabbits the catheter was removed after insertion for 3 days, and the course of the sterile endocarditis was studied at autopsy The average age of these rabbits was 189 2 days (range 176–207 days) and the average weight 3029 g (range 2820–3180 g) Three rabbits were sacrificed at 1 2, 3 4 10 20 and 30 days after removal of the catheter

TABLE 3 *Experimental Group III Long-term Study of Left Sided Sterile Endocarditis in Rabbits after Removal of Catheter The Catheter was Left in situ for 3 Days*

Number of rabbits	Interval between removal of catheter and sacrifice	Vegetations found		Weight of spleen (g)	Weight of heart (g)
10	60 days	None	Average	1 16	9 91
			SD	0 36	2.58

tions did not take place during the first 4 days. The vegetations decreased in size rapidly and 10 days after removal of the catheter only moderate oedema of the aortic valves and slight inflammation of the underlying myocardial tissue could be found.

Weight of the heart in the course of sterile endocarditis In rabbits with indwelling catheters, no noteworthy increase in weight occurred during observation for 5 days (Table 1). However in some rabbits observed for 60 days (Table 3), hypertrophy and enlargement of the heart were found. In four of 10 rabbits in this group, the weight had increased more than 10 g, while there was no increase in 21 rabbits in group I

Bacteriological examination No growth was obtained from any of the homogenized vegetations In rabbits which had no vegetations at the time of sacrifice, small pieces of aortic valves and myocardium were cultured, but gave no growth.

Pathology of Other Organs

The spleen appeared to be normal in all rabbits. The range of weight was 0 63–1 90 g Histological examination showed abundance of white pulp and some stasis In the kidneys, there were sterile, cortical, anaemic infarcts in five out of 21 rabbits which had indwelling catheters and had been sacrificed at regular intervals during the 5-day experimental period In rabbits from which the catheter was removed after insertion for 3 days, infarcts were found regularly (group II). The rabbits were sacrificed at intervals during 30 days observation In this group 24 of 42 kidneys had sterile infarcts, usually 2–5 mm^3 in size, and rarely up to 10 mm^3 None of the rabbits sacrificed after 60 days observation (group III) had fresh infarcts, but some had infarcts of older date, practically all scarred, The kidneys were found to be normal in size Gross examination of lungs, liver mesenteric glands and suprarenal glands showed normal findings in all rabbits, apart from some congestion Liquor in the pleural spaces and peritoneal cavity was found in few rabbits only that did not exceed 5 ml.

Bacteriological culture from blood and organs. No growth was obtained from any blood sample taken during the experimental periods. The organs were also found to be sterile, apart from sparse growth of *Bordetella bronchiseptica* from the lungs in a few rabbits.

Mortality and Body Weight

The sterile left-sided endocarditis was well tolerated in all rabbits Long-term observation of rabbits (group III) with sterile endocarditis, but without indwelling catheter showed that all animals survived in good condition up to the predetermined time of sacrifice (60 days). They sustained a moderate loss in weight between the first and second operation, followed by a significant increase. They gained on an average 7.0% (SE 1.6%) in weight from the first operation to autopsy and 9 8% (SE 1 8%) from the second operation to autopsy

DISCUSSION

The technique used by *Freedman* and co-workers (6 9 10) and by *Durack et al* (1 2) was blind insertion of a catheter either through the femoral vein, vena jugularis or the carotid artery into the right or left half of the heart. They showed that vegetations could be produced regularly by this procedure. Their later studies on infective endocarditis were performed by injection of bacteria into the blood stream of rabbits in which sterile vegetations had first been produced in this way and in most cases the catheter was left *in situ* during the infection (3 4 5 9). In spite of the simplicity and reproducibility of *Freedman*s and Durack s model, it appeared to us after some preliminary experiments that certain improvements were possible. By blind insertion, one cannot control the position of the tip of the catheter and therefore perforation of the myocardial wall sometimes occurs, as had also been observed by *Garrison & Freedman* (6). Furthermore, the production of vegetations varies somew

TABLE 1 *Experimental Group I Course of Left Sided Sterile Endocarditis in Rabbits with Indwelling Catheter*

Number of rabbits	Interval between insertion of catheter and sacrifice	Weight[a]) of vegetations (mg)			Average weight of	
					Spleen (g)	Heart (g)
3	3 h	0	0		1 43	8 13
3	12 h	0	0		1 00	8 13
3	1 day	8 5	8.5		1 00	8.05
3	2 days	12.2	14 4		1 49	9.22
3	3 days	19 4	20 6		1 26	8 89
3	4 days	27 8	48 7		1 23	8.17
3	5 days	45 4	115 2		1 10	8 29
21				Average	1 22	8 34
				SD	0.28	0.94

[a]) The weights are recorded for two rabbits only because the heart of one rabbit in each subgroup was taken for histological examination.

remained after 4 days, and they had almost disappeared after 10 days. Whitish fibrous changes in the left ventricle continued to be visible. The aortic valves appeared to be completely normal

Histological examination of the vegetations leaf lets and myocardial tissues Small disruptions of the endothelium covered by fibrin with mononuclear cells were the first signs of changes occurring 3–12 h after insertion of the catheter The sterile, fully developed vegetations were built up of a laminated material of fibrin and probably thrombocytes, always involving mononuclear cells. At the base of the vegetations the endothelium was disrupted, and oedema and reactive inflammatory response were found regularly in the underlying tissues. As early as 3 days after insertion of the catheter the appearance of small new vessels and proliferating fibroblasts was observed in the vegetations. Rapid acceleration of this process occurred during subsequent days and 4–5 days later development of collagen fibrilla was seen. After removal of the catheter (insertion for 3 days), the organization of the vegetations became pronounced, but definite endothelialization of the free surface of the vegeta

TABLE 2 *Experimental Group II Course of Left Sided Sterile Endocarditis in Rabbits after Removal of Catheter The Catheter was Left in situ for 3 Days*

Number of rabbits	Interval between removal of catheter and sacrifice (days)	Weight[a]) of vegetations (mg)			Average weight of	
					Spleen (g)	Heart (g)
3	1	28 3	35 2		1 00	7 03
3	2	28 7	26 3		0 97	7.59
3	3	28 3	14 5		1 28	8 58
3	4	5 0	5 0		0.70	8 19
3	10	0	0		0 78	8 30
3	20	0	0		1 00	8 43
3	30	0	0		1 00	9 58
21				Average	0 96	8 24
				SD	0.24	1 29

[a]) The weights are recorded for two rabbits only because the heart of one rabbit in each subgroup was taken for histological examination.

TABLE 3 *Experimental Group III Long-term Study of Left Sided Sterile Endocarditis in Rabbits after Removal of Catheter The Catheter was Left in situ for 3 Days*

Number of rabbits	Interval between removal of catheter and sacrifice	Vegetations found		Weight of spleen (g)	Weight of heart (g)
10	60 days	None	Average	1 16	9 91
			SD	0.36	2.58

tions did not take place during the first 4 days. The vegetations decreased in size rapidly and 10 days after removal of the catheter only moderate oedema of the aortic valves and slight inflammation of the underlying myocardial tissue could be found.

Weight of the heart in the course of sterile endocarditis. In rabbits with indwelling catheters, no noteworthy increase in weight occurred during observation for 5 days (Table 1). However in some rabbits observed for 60 days (Table 3), hypertrophy and enlargement of the heart were found. In four of 10 rabbits in this group, the weight had increased more than 10 g, while there was no increase in 21 rabbits in group I

Bacteriological examination. No growth was obtained from any of the homogenized vegetations. In rabbits which had no vegetations at the time of sacrifice, small pieces of aortic valves and myocardium were cultured, but gave no growth

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The spleen appeared to be normal in all rabbits. The range of weight was 0 63–1 90 g. Histological examination showed abundance of white pulp and some stasis. In the kidneys, there were sterile, cortical, anaemic infarcts in five out of 21 rabbits which had indwelling catheters and had been sacrificed at regular intervals during the 5-day experimental period. In rabbits from which the catheter was removed after insertion for 3 days, infarcts were found regularly (group II). The rabbits were sacrificed at intervals during 30 days observation. In this group 24 of 42 kidneys had sterile infarcts, usually 2–5 mm^3 in size, and rarely up to 10 mm^3. None of the rabbits sacrificed after 60 days observation (group III) had fresh infarcts, but some had infarcts of older date, practically all scarred. The kidneys were found to be normal in size. Gross examination of lungs, liver mesenteric glands and suprarenal glands showed normal findings *in all* rabbits, apart from some congestion. Liquor in the pleural spaces and peritoneal cavity was found in few rabbits only this did not exceed 5 ml.

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The sterile left-sided endocarditis was well tolerated in all rabbits. Long-term observation of rabbits (group III) with sterile endocarditis, but without indwelling catheter showed that all animals survived in good condition up to the predetermined time of sacrifice (60 days). They sustained a moderate loss in weight between the first and second operation, followed by a significant increase. They gained on an average 7 0% (SE 1 6%) in weight from the first operation to autopsy and 9.8% (SE 1 8%) from the second operation to autopsy

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The technique used by *Freedman* and co-workers (6 9 10) and by *Durack et al* (1 2) was blind insertion of a catheter either through the femoral vein, vena jugularis or the carotid artery into the right or left half of the heart. They showed that vegetations could be produced regularly by this procedure. Their later studies on infective endocarditis were performed by injection of bacteria into the blood stream of rabbits in which sterile vegetations had first been produced in this way and in most cases the catheter was left *in situ* during the infection (3 4 5 9). In spite of the simplicity and reproducibility of *Freedman's* and Durack's model, it appeared to us after some preliminary experiments that certain improvements were possible. By blind insertion, one cannot control the position of the tip of the catheter and therefore perforation of the myocardial wall sometimes occurs, as had also been observed by *Garrison & Freedman* (6). Furthermore, the production of vegetations varies somew

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Number of rabbits	Interval between insertion of catheter and sacrifice	Weight[a]) of vegetations (mg)		Average weight of	
				Spleen (g)	Heart (g)
3	3 h	0	0	1.43	8.13
3	12 h	0	0	1.00	8.13
3	1 day	8.5	8.5	1.00	8.05
3	2 days	12.2	14.4	1.49	9.22
3	3 days	19.4	20.6	1.26	8.89
3	4 days	27.8	48.7	1.23	8.17
3	5 days	45.4	115.2	1.10	8.29
21			Average	1.22	8.34
			SD	0.28	0.94

[a]) *The weights are recorded for two rabbits only because the heart of one rabbit in each subgroup was taken for* histological examination.

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TABLE 2 *Experimental Group II Course of Left Sided Sterile Endocarditis in Rabbits after Removal of Catheter The Catheter was Left* in situ *for 3 Days*

Number of rabbits	Interval between removal of catheter and sacrifice (days)	Weight[a]) of vegetations (mg)		Average weight of	
				Spleen (g)	Heart (g)
3	1	28.3	35.2	1.00	7.03
3	2	28.7	26.3	0.97	7.59
3	3	28.3	14.5	1.28	8.58
3	4	5.0	5.0	0.70	8.19
3	10	0	0	0.78	8.30
3	20	0	0	1.00	8.43
3	30	0	0	1.00	9.58
21			Average	0.96	8.24
			SD	0.24	1.29

[a]) The weights are recorded for two rabbits only because the heart of one rabbit in each subgroup was taken for *histological examination.*

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hat, depending on the level at which the tip is positioned. These disadvantages can be circumvented successfully by using the technique described in the present paper The guide wire and the distance markings on the catheter ensure precise positioning of the tip so that unintentional damage can be avoided and a more uniform localization of the vegetations can be obtained.

As it would seem desirable to avoid disturbing influence of the indwelling catheter during the infectious processes we also decided to ascertain whether the continued presence of the catheter was indispensable for the development of the infection. However it was first necessary to find the time for withdrawal of the catheter that would still permit the regular development of an infection, and to do this the spontaneous course of the non infected processes was examined in detail, as reported in the present paper Since it was found that not until after 3 days had the vegetations reached a suitable degree of development in all rabbits and as the histological examination indicated that organization and endothelialization at that time were still negligible it was decided to withdraw the catheter after 3 days and then introduce the bacteria. To wait for 3 days had the further advantage that the animals did not have to be allocated to different experimental groups until it was known which animals had survived the operation.

The histological appearance of the vegetations was found to be similar to that found by *Perlman & Freedman* (9) and by *Durack et al.* (2). However we also observed that the sterile vegetations were polypous and aciniform in the aorta and on the aortic valves, while the vegetations in the ventricle were rather flat and smooth It seems conceivable that the bacterial aggregations on and the growth in these two types of vegetations might be different.

While the presence of the catheter leads to continuous enlargement of the sterile vegetations, a surprisingly rapid disappearance of the vegetations was observed after withdrawal of the catheter Histological studies showed that the sterile vegetations had disappeared almost completely before the completion of restorative processes, *i.e* newbuilding of vessels and endothelialization. Fibrinolytic activity probably plays an essential part but also mechanical tearing must be a contributing factor as evidenced by the regular occurrence of kidney infarcts

Rabbits with long-standing sterile endocarditis appeared to be unaffected Even the infarction of the kidneys did not seem to be harmful. However some of the rabbits developed hypertrophy of the heart, as also observed by *Durack et al* (2) The aortic valves were found to be entirely normal in these rabbits, and none of them had signs of haemodynamic disturbances. The significant increase in body weight also indicates that sterile endocarditis is harmless and self-limiting in rabbits and has hardly any influence on the survival.

With the aid of this model it would seem possible to perform evaluation of the pathogenicity of different bacterial species by measuring the time of survival.

We are indebted to the staff of the *Institute for Experimental Research in Surgery University of Copenhagen*, for advice and assistance in performing the preliminary investigations, and to *K L. Fennestad*, V.M D Statens Seruminstitut, for provision and maintenance of the rabbits. We are grateful to Mrs. *U A S Petersen* for technical assistance and to Miss *A Overgaard* and Mr *F Laursen* for photographic assistance.

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EXPERIMENTAL ENDOCARDITIS IN RABBITS

2 *Course of Untreated* Streptococcus faecalis *Infection*

ERNÖ GUTSCHIK and NILS CHRISTENSEN

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Gutschik, E. & Christensen, N. Experimental endocarditis in rabbits 2 Course of untreated *Streptococcus faecalis* infection Acta path microbiol scand Sect B, 86 223–228 1978

Insertion of a polyethylene catheter in the left side of the heart was used to induce sterile endocarditis in 34 rabbits. Bacterial endocarditis was established by injection of approximately 10^8 *Streptococcus faecalis* into the blood stream simultaneous with the removal of the catheter which had been in place for 3 days. The course of the bacterial endocarditis was examined by autopsy of rabbits sacrificed at regular intervals after the infection. The results showed that the presence of the catheter was not essential for the induction or maintenance of the infection. Growth of the bacteria took place in the preformed vegetations in the aorta, on the aortic valves and in the left ventricle. However increases in the size of the vegetations, a high density of bacteria in the vegetations and secondary spreading were found only on the aortic valves. The extracardial manifestations of left-sided *S. faecalis* endocarditis included constant bacteriaemia, a high frequency of septic kidney infarcts and enlargement of the spleen. This form of experimentally provoked bacterial endocarditis in rabbits provides a good imitation of human subacute endocarditis, and would thus seem to be suitable for further study of the pathophysiology of endocarditis and evaluation of the effect of treatment with antibiotics

Key words Experimental endocarditis, bacterial endocarditis, rabbits, *Streptococcus faecalis*

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A reproducible model of experimental endocarditis in rabbits was described by *Garrison & Freedman* in 1970 (6) The method is based on the observation that a polyethylene catheter installed in the inner heart results in the development of sterile vegetations. It was shown in a previous report that a uniform development of such vegetations can be ensured by means of an improved left-sided catheterization technique. This provides the possibility of establishing bacterial endocarditis, even when the catheter is removed after insertion for 3 days (8)

The bacterial endocarditis can be induced either by introducing a bacterial suspension through the catheter (6 12, 13) or by injecting the bacteria into the blood stream at various times after insertion of the catheter (2 3 4 5 9 15). A rapid fatal course has been observed in rabbits with left-sided endocarditis, while in right-sided endocarditis the course was more varied and spontaneous recovery occurred (1 4 6). Removal of the catheter at various times after insertion resulted in a considerable increase in the frequency of spontaneous recovery particularly in rabbits with the right-sided disease (1 4 13), but also in those with endocarditis on the left side (1 13)

The aim of this study was to examine the development and course of bacterial endocarditis in rabbits inoculated intravenously with *Streptococcus faecalis* simultaneously with removal of the catheter after insertion for 3 days. The data presented are obtained by autopsy of rabbits sacrificed at regular intervals after the infection.

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Insertion of a polyethylene catheter in the left side of the heart was used to induce sterile endocarditis in 34 rabbits. Bacterial endocarditis was established by injection of approximately 10^8 *Streptococcus faecalis* into the blood stream simultaneous with the removal of the catheter which had been in place for 3 days. The course of the bacterial endocarditis was examined by autopsy of rabbits sacrificed at regular intervals after the infection. The results showed that the presence of the catheter was not essential for the induction or maintenance of the infection. Growth of the bacteria took place in the preformed vegetations in the aorta, on the aortic valves and in the left ventricle. However increases in the size of the vegetations, a high density of bacteria in the vegetations and secondary spreading were found only on the aortic valves. The extracardial manifestations of left-sided *S. faecalis* endocarditis included constant bacteraemia, a high frequency of septic kidney infarcts and enlargement of the spleen. This form of experimentally provoked bacterial endocarditis in rabbits provides a good imitation of human subacute endocarditis, and would thus seem to be suitable for further study of the pathophysiology of endocarditis and evaluation of the effect of treatment with antibiotics.

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The bacterial endocarditis can be induced either by introducing a bacterial suspension through the catheter (6, 12, 13) or by injecting the bacteria into the blood stream at various times after insertion of the catheter (2, 3, 4, 5, 9, 15). A rapid fatal course has been observed in rabbits with left-sided endocarditis, while in right-sided endocarditis the course was more varied and spontaneous recovery occurred (1, 4, 6). Removal of the catheter at various times after insertion resulted in a considerable increase in the frequency of spontaneous recovery particularly in rabbits with the right-sided disease (1, 4, 13), but also in those with endocarditis on the left side (1, 13).

The aim of this study was to examine the development and course of bacterial endocarditis in rabbits inoculated intravenously with *Streptococcus faecalis* simultaneously with removal of the catheter after insertion for 3 days. The data presented are obtained by autopsy of rabbits sacrificed at regular intervals after the infection.

TABLE 1 *Course of Left sided* Streptococcus faecalis *Endocarditis in 34 Rabbits. The Catheter was Removed after Insertion for 3 Days, Simultaneously with the Intravenous Injection of 10⁸ Bacteria*

Number of rabbits	Interval between removal of catheter and sacrifice (hours)	Average weight of			Average of colony forming unit	
		Vegetations[a]) (mg)	Spleen (g)	Heart (g)	per g vegetation	per ml blood
3	1½	41 0	1 22	6 94	$3\,3 \times 10^5$	$1\,1 \times 10^1$
4	3	38 9	1 03	8 12	$8\,3 \times 10^5$	$7\,4 \times 10^0$
4	6	34 4	1 19	7 92	1.8×10^7	1.3×10^1
4	12	29 9	1 30	8 05	$4\,3 \times 10^8$	$1\,2 \times 10^1$
4	24	51 7	1 98	8.26	$4\,9 \times 10^9$	$5\,9 \times 10^1$
4	48	52.4	1.53	8 07	$1\,3 \times 10^{10}$	$1\,7 \times 10^2$
4	72	90 0	3 26	8 71	2.1×10^{10}	1.6×10^4
4	96	153 9	3 52	8 24	2.8×10^{10}	$9\,3 \times 10^3$
3	56–72 (succumbed spontaneously)	135 3[b])	2 93	8.99	$4\,6 \times 10^{10}$	–

[a]) Data recorded for 3 rabbits only since the heart of one rabbit in each subgroup was used for histology
[b]) Data recorded for 2 rabbits

heart were observed in one rabbit, but none of the animals had vegetations in the right side. The number of bacteria increased rapidly in the vegetations. There were about 10^5 bacteria per g vegetation 1½ hours after the inoculation, increasing to 10^8 to 10^9 bacteria per g after 24 hours (Table 1). Within 2 days the number of bacteria in the vegetations had reached a stationary level.

Weight of heart. Even though dilatation and some hypertrophy of the wall of the heart was seen in a number of rabbits, no definite trend in the weight was noted during the experiment (Table 1). There was some variation in the weight of the heart between 5 87 and 10.53 g in individual rabbits.

Histology of vegetations. The vegetations consisted of an amorphous eosinophilic mass of fibrin and thrombocytes, with some indication of stratification. In addition to mononuclear cells, they contained granulocytes, though there were not many of these and they were never in direct contact with the bacteria. Microscopically the earliest finding of bacteria was 12 hours after the infection (Figs 5 and 6).

There was a pronounced difference in the number of bacteria in the vegetations at various sites in the left half of the heart. While the bacteria occurred in the form of large masses of oval or circular rings in the vegetations on the aortic valves (Fig 7), similar concentrations were rare in the vegetations from the left ventricle and aortic wall during the first 24 hours of infection and no bacteria could be demonstrated after this time. The bacteria were always embedded in the fibrin substance itself and never extended to the surface of the vegetations (Fig. 5 6 7). Severe reactive inflammation and oedema were found in the underlying tissue, but the bacteria did not invade these structures. Healing processes with ingrowing vessels and fibroblasts were only present to a slight degree.

Extracardial Manifestations of Bacterial Endocarditis

Spleen. A characteristic change in the colour of the spleen from light red to dark red could be seen during the infection period. In some rabbits the parenchyma was hyperaemic with increased white pulpa. The weight of the spleen during the first 48 hours was 0 62 to 1 74 g, except in one rabbit where it was 3 04 g. After 56 hours an increase in the weight occurred to > 2 g (2.40 to 5.54 g) except in one rabbit where it was 1 70 g (Table 1).

Kidneys. Anaemic and mainly cortical infarcts were found regularly. The frequency of the fresh infarcts increased with the survival time. Thus 27 of 46 kidneys had one or more infarcts during the first 48 hours, while 20 of 22 kidneys had infarcts after that time. The size of the infarcts also increased during the infection from 2 to 5 mm² in the early phase to over 10 mm in the later phase.

Other organs. The lungs were oedematous and small atelectases were often seen. Microscopy of the liver showed diffuse focal cell necrosis in the majority of the rabbits and sinus reticulosis was found in the mesenteric glands. The suprarenal glands were normal both macroscopically and microscopically.

MATERIALS AND METHODS

The experimental animals were random-bred, male albino rabbits (Ssc:CPH) from Statens Seruminstitut. The average weight was 2979 g (range 2630–3240) and the average age 181.2 days (range 135–226 days).

Pretreatment for producing left sided sterile endocarditis. The rabbits were anaesthetized with mebumal natrium intravenously. The average dose at the first operation was 23.0 mg (SD 2.4) and at the second operation 22.4 mg (SD 2.5). A polyethylene catheter was inserted into the left ventricle of the heart, using the technique described previously (8). The catheter was left in place for 3 days.

Infecting organism. The strain of *S. faecalis* used in all infection experiments was originally recovered from the blood of a patient with bacterial endocarditis. The most important tests used in identification were the ability to grow in 6.5% NaCl broth, growth at 10° and 45°C and reduction of 0.04% potassium tellurite. Furthermore, the strain was β-haemolytic and able to liquefy gelatine after incubation for 24 hours at 22°C. The media and tests used for subcultivation and identification have been described in detail previously (7, 11).

Identification of the organisms recovered from blood, vegetations and different organs from the experimental animals by subculture on 5% blood agar plates was made by microscopy of typical colonies and by tests for tellurite resistance and gelatine liquefaction.

Infection of pretreated animals. After 3 days with indwelling catheter, a second operation was carried out to remove the catheter and 1 ml of a bacterial suspension of *S. faecalis* was simultaneously injected into a marginal ear vein. The suspension was prepared from an overnight culture on 5% blood agar suspended in saline. This suspension contained approximately 10^8 colony forming units (CFU) per ml (mean 1.299×10^8, SEM 0.036×10^8).

Quantitative blood culture. Blood specimens were taken from all rabbits just before the predetermined time of sacrificing. One or 2 ml blood was drawn from the marginal ear vein into a syringe previously rinsed with 0.9% saline containing 10 U Heparin® (Leo Laboratories, Copenhagen) per ml. One ml was streaked immediately onto 5% blood agar plates (diameter 13.5 cm). When a large number of CFU was expected, a further 1 ml blood was diluted in 0.9% saline 1:10, and 1 ml of this was streaked on blood agar plates. The plates were incubated at 35°C and the number of colonies was counted 48 hours later, since after that time no further development of new colonies had been observed.

Autopsy. All cages were inspected at least every 6 hours day and night, so that in cases of spontaneous death the animals could be removed to a refrigerator within 6 hours and kept there until autopsy could be performed.

The heart and spleen were weighed, and fluid from the pleural spaces and peritoneal cavity was collected and measured. The vegetations from the left side of the heart were removed aseptically, weighed, added to ten volumes saline per unit weight vegetation and homogenized in a glass tissue grinder. To determine the efficacy of homogenization, gram-stained slides were prepared from each suspension. Quantitative culture was made from ten-fold dilution in saline and from the undiluted suspension by spreading triplicate 0.1 ml samples on blood agar plates. The colonies were counted after incubation for 24 hours at 35°C, and the number of organisms was expressed as CFU/g wet tissue.

Semiquantitative culture was carried out routinely from the peritoneal cavity, liver, spleen, kidneys, mesenteric glands, pleural spaces and lungs by seeding blood agar plates with small pieces of tissue or fluid taken with a small platinum loop from the peritoneal cavity and the pleural spaces. After incubation for 48 hours at 35°C, the degree of growth was read as 0, +, + + and + + +, corresponding to no growth, from 1 to 10 colonies, from 11 colonies to confluent growth and confluent growth. The heart of one rabbit from each of the subgroups in the experiment (Table 1) was used exclusively for the histological examination, together with the liver, lungs, spleen, mesenteric glands and suprarenal glands from the same rabbit.

General plan of the study. A total of 34 rabbits was used. Three rabbits were sacrificed 1½ hours after intravenous injection of bacteria, and groups of four rabbits after 3, 6, 12, 24, 48, 72 and 96 hours. Three rabbits which succumbed spontaneously between 56–72 hours after infection are grouped separately (Table 1).

RESULTS

Bacterial endocarditis was demonstrated in all rabbits by culture of *S. faecalis* from vegetations, except in the case of rabbits which died spontaneously, where the diagnosis of bacterial endocarditis was established by histological examination of vegetations. Spontaneous death occurred in three of the 34 rabbits 56 to 72 hours after the intravenous injection of bacteria (Table 1).

Cardial Manifestations of Bacterial Endocarditis

Distribution, weight and bacteriology of vegetations. During the early phase of the infection, the vegetations were found regularly on one or two of the aortic valves, on the wall of the aorta a few millimetres above the valves, at the base of the papillary muscles and at the bottom of the left ventricle (Figs 1 and 3). The vegetation showed decreasing weight during the first 12 hours, but the weight increased during the later course (Table 1). There were signs of secondary spreading, as shown by development of vegetations on all three aortic valves, and also in some rabbits by the development of small flat vegetations on the wall of the aorta. Parallel with the increasing growth of vegetations on the aortic valves, there was a decrease in the size and extent of the vegetations in the left ventricle and on the wall of aorta (Figs 1, 2, 3 and 4). Vegetations on the atrioventricular valves in the left side of the

TABLE 1 *Course of Left-sided Streptococcus faecalis Endocarditis in 34 Rabbits. The Catheter was Removed after Insertion for 3 Days, Simultaneously with the Intravenous Injection of 10^7 Bacteria*

Number of rabbits	Interval between removal of catheter and sacrifice (hours)	Average weight of			Average of colony forming units	
		Vegetations*) (mg)	Spleen (g)	Heart (g)	per g vegetation	per ml blood
3	1½	41.0	1.22	6.94	3.3×10^5	1.1×10^1
4	3	38.9	1.03	8.12	8.3×10^5	7.4×10^0
4	6	34.4	1.19	7.92	1.8×10^7	1.3×10^1
4	12	29.9	1.30	8.05	4.3×10^8	1.2×10^1
4	24	51.7	1.98	8.26	4.9×10^9	5.9×10^1
4	48	52.4	1.53	8.07	1.3×10^{10}	1.7×10^2
4	72	90.0	3.26	8.71	2.1×10^{10}	1.6×10^4
4	96	153.9	3.52	8.24	2.8×10^{10}	9.3×10^3
3	56–72 (succumbed spontaneously)	135.3#)	2.93	8.99	4.6×10^{10}	–

*) Data recorded for 3 rabbits only since the heart of one rabbit in each subgroup was used for histology

#) Data recorded for 2 rabbits

heart were observed in one rabbit, but none of the animals had vegetations in the right side. The number of bacteria increased rapidly in the vegetations. There were about 10^5 bacteria per g vegetation 1½ hours after the inoculation, increasing to 10^8 to 10^9 bacteria per g after 24 hours (Table 1). Within 2 days the number of bacteria in the vegetations had reached a stationary level.

Weight of heart. Even though dilatation and some hypertrophy of the wall of the heart was seen in a number of rabbits, no definite trend in the weight was noted during the experiment (Table 1). There was some variation in the weight of the heart between 5.87 and 10.53 g in individual rabbits.

Histology of vegetations. The vegetations consisted of an amorphous eosinophilic mass of fibrin and thrombocytes, with some indication of stratification. In addition to mononuclear cells, they contained granulocytes, though there were not many of these and they were never in direct contact with the bacteria. Microscopically the earliest finding of bacteria was 12 hours after the infection (Figs 5 and 6).

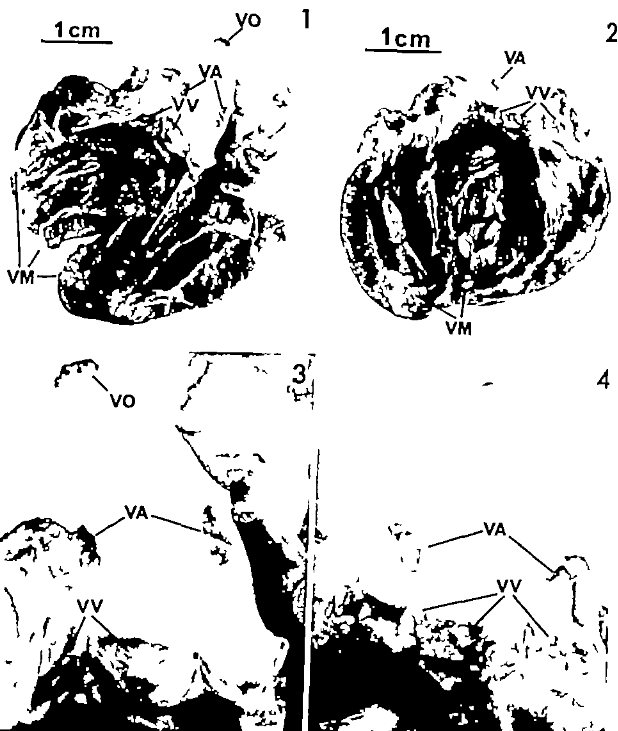
There was a pronounced difference in the number of bacteria in the vegetations at various sites in the left half of the heart. While the bacteria occurred in the form of large masses of oval or circular rings in the vegetations on the aortic valves (Fig. 7), similar concentrations were rare in the vegetations from the left ventricle and aortic wall during the first 24 hours of infection and no bacteria could be demonstrated after this time. The bacteria were always embedded in the fibrin substance itself and never extended to the surface of the vegetations (Figs 5, 6, 7). Severe reactive inflammation and oedema were found in the underlying tissue, but the bacteria did not invade these structures. Healing processes with ingrowing vessels and fibroblasts were only present to a slight degree.

Extracardial Manifestations of Bacterial Endocarditis

Spleen. A characteristic change in the colour of the spleen from light red to dark red could be seen during the infection period. In some rabbits the parenchyma was hyperaemic with increased white pulpa. The weight of the spleen during the first 48 hours was 0.67 to 1.74 g, except in one rabbit where it was 3.04 g. After 56 hours an increase in the weight occurred to > 2 g (2.40 to 5.54 g), except in one rabbit where it was 1.70 g (Table 1).

Kidneys. Anaemic and mainly cortical infarcts were found regularly. The frequency of the fresh infarcts increased with the survival time. Thus 27 of 46 kidneys had one or more infarcts during the first 48 hours, while 20 of 22 kidneys had infarcts after that time. The size of the infarcts also increased during the infection from 2 to 5 mm^2 in the early phase to over 10 mm^2 in the later phase.

Other organs. The lungs were oedematous and small atelectases were often seen. Microscopy of the liver showed diffuse focal cell necrosis in the majority of the rabbits and sinus reticulosis was found in the mesenteric glands. The suprarenal glands were normal both macroscopically and microscopically.



Figures 1–4 are photographs of the left side of the rabbit s heart. Figures 5–7 are gram-stained sections of the aortic valves The following abbreviations are used throughout V Vegetations; VV Vegetations on the aortic Valves, VA Vegetations above the Aortic valves, VO Vegetations at the Orifice of a anonyma, VM Vegetations on the wall of Myocardium; AV Aortic Valve; BA Bacterial Aggregation; E, Erythrocytes, L, Leucocytes.

Fig 1 View of left ventricle and ascending aorta showing the characteristic distribution of vegetations 24 hours after inoculation with bacteria.

Fig 2 View of heart showing left ventricle and ascending aorta showing the characteristic distribution of vegetations 48 hours after inoculation with bacteria.

Fig 3 A closer view of the aortic valve and ascending aorta seen in Fig. 1 ×3

Fig 4 A closer view of the aortic valve and ascending aorta seen in Fig. 2, ×3



Fig 5 A section through a cusp of the aortic valve showing a vegetation 12 hours after inoculation with bacteria, × 29



Fig 6 A closer view of the vegetation seen in Fig 5 showing an aggregation of bacteria with only a slight inflammatory reaction on the adjacent valve leaflet, × 276

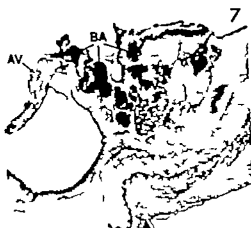


Fig 7 A large vegetation on the valve leaflet 96 hours after inoculation with bacteria Note the increase in the number of bacteria aggregates, × 40

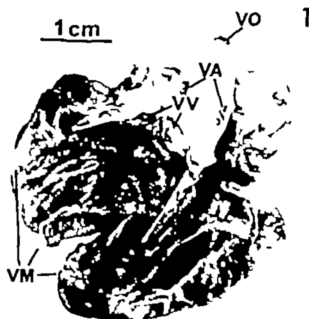
It was seldom possible to demonstrate bacteria in the organs by microscopy except for the kidneys, where they could be found in areas with infarcts.

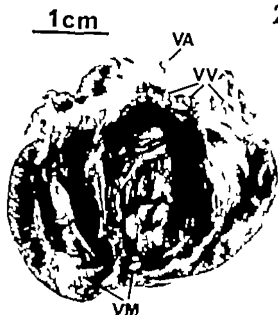
Culture from blood and organs. During the first 48 hours after infection, the number of bacteria per ml blood was small, but after 72 hours all the rabbits had bacteraemia with 10^3 to 10^4 bacteria per ml blood (Table 1) Growth of *S. faecalis* occurred regularly from the spleen and the liver and to a lesser extent from the lungs at 1 ½ and 6 hours after inoculation of the bacteria. Between 6 hours and 48 hours after inoculation, growth was less pronounced or absent, while after 48 hours there was again strong growth from spleen, liver and kidneys, and also from the mesenteric glands to some extent. The pleural spaces and peritoneal cavity were sterile in all cases and there was no accumulation of fluid. A few ml pericardial exudate with strong growth of *S faecalis* were found in two of three of the spontaneously dead rabbits.

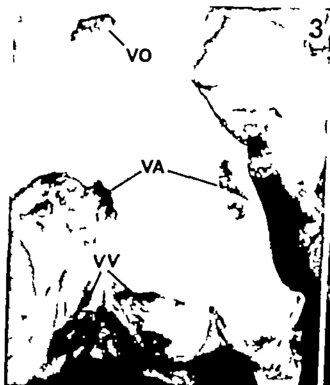
DISCUSSION

The study has shown that the presence of the catheter is not essential for the regular induction of *S faecalis* endocarditis, nor is the continued presence of the catheter necessary for maintenance of the infection. It would appear that *S faecalis* endocarditis provoked by this means has a fatal issue, as also observed in a yet unpublished study involving 100 rabbits. These results differ from certain previous reports in which experimental right-sided endocarditis with *S viridans* (4), and maintenance of left-sided endocarditis with *Staphylococcus aureus* (13) and *Pseudomonas aeruginosa* (1) were conditional on the continued presence of the catheter during the infection However the results are in accordance with other studies which showed that left-sided endocarditis could be maintained by *S viridans* (14), *S sanguis* (10) and *S faecalis* (15), even though the catheter was removed. According to the literature, the presence of the catheter during the infection provokes a more severe course of the disease, thus causing it to resemble human subacute bacterial endocarditis to a lesser extent and making it less suitable for experimental treatment.

The present study shows that the continuous deposit of fibrin, resulting in the development of vegetations is associated with continued growth of the bacteria. This is in strong contrast to the rapid elimination of the sterile vegetations after removal of the catheter (8). Some special features must pertain to the vegetations on the aortic valves, since the density of the bacteria was much greater and the vegetations larger than on the wall of the left ventricle, even though endothelial damage and preformed sterile vegetations are known also to occur regularly on the wall of the left ventricle (8)









Figures 1–4 are photographs of the left side of the rabbit s heart. Figures 5–7 are gram-stained sections of the aortic valves The following abbreviations are used throughout V Vegetations, VV Vegetations on the aortic Valves, VA, Vegetations above the Aortic valves, VO Vegetations at the Orifice of a. anonyma, VM Vegetations on the wall of Myocardium, AV Aortic Valve; BA, Bacterial Aggregation, E, Erythrocytes, L, Leucocytes.

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Fig 4 A closer view of the aortic valve and ascending aorta seen in Fig. 2, × 3

Acta path. microbiol. scand. Sect. B, 86: 229–236, 1978

RABBIT POLYMORPHONUCLEAR LEUKOCYTE CHEMOTACTIC FACTOR GENERATED *IN VIVO* BY *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE

I. Isolation and Physico-Chemical Characterization

KJELL SVEEN

Sveen, K. Rabbit polymorphonuclear leukocyte chemotactic factor generated *in vivo* by *Bacteroides fragilis* lipopolysaccharide. I. Isolation and physico-chemical characterization. Acta path. microbiol. scand. Sect. B, 86: 229–236, 1978.

By chromatographic separation on Sephadex gels a peptide, termed the lipopolysaccharide-induced chemotactic factor (LPS-CF), has been isolated from inflammatory exudate. The exudate was obtained from Teflon® chambers implanted subcutaneously in rabbits 3 h after LPS from *Bacteroides fragilis* ss. *fragilis* had been injected. Three chemotactic peaks were eluted by fractionation of the exudate on Sephadex G-200 columns, one major peak with molecular weight of approximately 16,000 and two minor peaks with molecular weights of approximately 68,000 and 7,000. Refiltration of the major peak on G-75 showed the same elution profile as that found on G-200 columns. By addition of 8 M urea to the elution fluid only the major and the low molecular weight peaks appeared. The molecular weight of the major chemotactic peak was calculated to 16,000 on Sephadex gels, and also using SDS-polyacrylamide gel electrophoresis and equilibrium centrifugation. The chemotactic factor was quite heat-stable and was also non-dialyzable, and freezing and thawing as well as storage at 4° C for several weeks did not impede its activity. This chemotactic factor is probably identical to the cytotoxic fragment split off from C5 upon interaction with LPS.

Key words: *B. fragilis*; lipopolysaccharide; exudate; gel filtration; chemotactic factor; molecular weight determination.

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An important factor in the accumulation of polymorphonuclear leukocytes (PMNs) in the response to the endotoxic cell wall lipopolysaccharide (LPS) of Gram-negative bacteria has been thought to be complement (C) fragments (17, 18, 19). Thus, the major chemotactic principle which attracts PMNs upon incubation of LPS, immune complexes or a cobra venom factor with normal sera, has been shown to be attributed to a split product of the fifth complement factor (15, 17, 19). LPS activates the complement system through the alternative complement pathway (3, 4, 23). Besides inducing the generation of cytotoxins, which act directly on the leukocytes (8), other mediators of inflammation such as opsonins (13) and anaphylatoxins (9, 10) are also elaborated.

The usefulness of wound chambers implanted subcutaneously in normal rabbits and mice, as well as in mice genetically deficient in C5, has led to a series of experiments in this laboratory on the migration of PMNs in response to LPS. Thus LPS from *Bacteroides fragilis* ss. *fragilis* also has been found to be an active attractant on PMNs *in vivo* (22) as well as *in vitro* (21), although its endotoxic activity has been questioned by others (7). This chemotactic capacity, however, is latent and is dependent on complement for the generation of cytotaxins. The present study is concerned with both the isolation and the physico-chemical charac-

What these special features are is not known at present.

The histological picture of the septic vegetations is the same as that described by *Garrison & Freedman* (6) and *Durack & Beeson* (3), despite the fact that they used other microorganisms. The results of the present study show that the healing processes in the infected vegetations occurred later and were less complete than in the sterile vegetations (8) and showed no signs of endothelialization. In conjunction with the high frequency of septic infarcts these results indicate that a dynamic process is taking place in the infected vegetations including among other phenomena the frequent mechanical separation of small clumps of the vegetations, continued deposition of fibrin and persistent bacterial growth.

Bacteriaemia would seem to be a valuable parameter indicating that bacterial endocarditis had developed and monitoring the course of the infection. On the other hand, the growth of bacteria in the various organs would seem only to reflect the degree of bacteriaemia and is presumably conditional on the content of blood in the organs. This is in agreement with the observation of *Durack & Beeson* (3). Histological examination of the organs has not revealed pathological processes except for the kidney infarcts which must be assumed to have had some effect on the course of the endocarditis or the survival of the rabbits.

The data presented show that bacterial endocarditis can be induced with certainty in rabbits, even though the catheter is removed simultaneously with the intravenous inoculation of the bacteria. The experimental model would therefore seem to provide a good imitation of human endocarditis and to be suitable for studies of the pathophysiology of endocarditis and evaluation of the effect of antibiotics.

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Gas Liquid Chromatography

Amino acid analyses Samples of 1 mg of the pooled, dialyzed and lyophilized LPS-CF were hydrolysed in 6 N HCl at 105° C for 18 h and prepared for analyses by gas liquid chromatography according to *Rasch & Gehrke* (18)

Analysis of aldoses and hexosamines The preparing of samples of the LPS-CF for the detection of aldoses and hexosamines, as well as the analysis of the samples, was performed as described previously (20)

Rabbit Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMNs) were harvested from the peritoneal cavity of New Zealand White rabbits 8–10 h after local injection of 200 ml of a solution containing 0 1 per cent glycogen (E Merck AG Darmstadt, W-Germany) in sterile saline Garamycin® and fungizone® were added in amounts of 50 and 2 5 μg per ml of the glycogen solution, respectively The harvesting and the isolation of the cells was performed as described earlier (21) After the isolation, the PMNs were resuspended in Gey's medium containing antibiotics in amounts equal to those in the glycogen solution The Gey's medium was then adjusted to 2 per cent (w/) solution with BSA (Armour Pharmaceutical Co Ltd), one ml of the PMN suspension containing approximately 10^7 cells

Measurement of Chemotaxis

The migration of PMNs was determined by the Boyden Micropore technique (2) using a modified Boyden chamber (19) (Neuroprobe, Bethesda, Md USA) The upper compartment of the chamber received 4 10^4 cells These were centrifuged down on a Millipore membrane (Neuroprobe) with pore size of 3 μ before placing the membrane in the chamber (21) The lower compartment contained the test medium The chambers were incubated at 37° C in humid air for 3 h Before the filters were removed, fixed, stained and mounted as previously stated (21), the cells on the upper surface of the filters were carefully sucked off Five fields of the lowermost surface of the filters were selected for counting at magnification of X320 with the aid of a micrograd (Leitz Wetzlar 10 × 10 mm)

EXPERIMENTS AND RESULTS

In preliminary experiments inflammatory exudates aspirated from the chambers at different time intervals after LPS injection, were fractionated on Sephadex G-200 columns and the eluate tested for chemotactic activity in modified Boyden chambers In these experiments the highest activity was found in the eluate after fractionation of exudate aspirated 3 h after local injection of LPS This exudate was, therefore, gel-filtered in order to isolate the LPS-induced PMN chemotactic factor

Aliquots of 4 ml exudate were passed through G-200 columns. The major chemotactic peak was always found in the 16,000 MW region (K_{av} 0 71), slightly ahead of the cytochrome C marker (Fig. 1). Slighter but still significant chemotactic activity was retained in the eluate with peaks corresponding to K_{av} 0 41 (MW 68,000) and K_{av} 0 92 (MW 7,000) Invariably a fourth peak with K_{av} of 0 08 indicative of a MW of 300,000 was found. No significant activity could be found in the void volume or in the eluate of non-stimulated exudate

Collected fractions of the major peak corresponding to a MW range of 12 000 to 20 000 were dialyzed, lyophilized and pooled This pooled sample was reconstituted with eluent buffer to a volume of 0.5 ml and applied to the Sephadex G-75 column. In these experiments, three peaks of chemotactic activity were constantly found in the eluate (Fig 1) These peaks showed the same pattern as those found in the G-200 eluate with respect to MW and mutual chemotactic activity The major peak corresponding to a MW of 16,000 (K_{av} 0.37), and that of the two other peaks were calculated to 7 000 (K_{av} 0.56) and 68 000 (K_{av} 0 05). No activity could be detected in the void volume. There was no significant change in the chemotactic activity of the fractions before and after dialysis

In another experiment, the collected pooled fractions of the major chemotactic peak eluted on G-200 columns were re-passed over a Sephadex G 75 column, equilibrated and eluted with 8 M urea in PBS. After dialysis of the collected fractions they were examined for chemotactic capacity on rabbit peritoneal PMNs No activity was found either in the void volume or in the MW region corresponding to 68,000 Furthermore, the chemotactic peak with a MW of 7,000 now exhibited almost the same chemotactic activity as the peak eluted slightly behind the myoglobin marker (Fig 2) For characterization and further study of the chemotactic factor only the central fractions of the major peak eluted without urea on Sephadex G-75 columns were used.

The MW of the major chemotactic peak (LPS-CF) in ten G-75 runs calculated from proteins of known molecular weights was 16,000 with a standard deviation of 470 In equilibrium centrifugation the MW of the LPS-CF calculated from two runs was 16 000 In polyacrylamide gel electrophoresis, a single band was seen after protein staining. In SDS-polyacrylamide gel electrophoresis the LPS-CF moved between the cytochrome C and the light IgG chain, and its MW was determined to be 16 400 when the moving front of the protein bands were used for calculation (Fig. 3).

The results of the quantitative amino acid analysis are summarized in Table 1 Hexosamines or neutral sugars were not detected. The mean

teristics of the PMN chemotactic factor present in the inflammatory exudate after injection of *Bacteroides fragilis* LPS into wound chambers implanted in rabbits.

MATERIALS AND METHODS

Source and Preparation of LPS

LPS (endotoxin) was isolated from *Bacteroides fragilis* subspecies *fragilis* strain Lille E 323 by phenol water extraction (27) Further purification of the LPS by ultracentrifugation and treatment of the pellet with ribonuclease and deoxyribonuclease has been reported previously (20) Stock suspensions of LPS containing 2.5 mg per ml was made in sterile isotonic saline. This was treated ultrasonically (MSE/Mullard 60W 20 kc/s) at 0° C for 2 min.

Inflammatory Exudate

Five Teflon® chambers (12) were implanted subcutaneously on each lateral side of the abdomen of six months-old New Zealand White rabbits as described before (24). Six days after implantation the exudate was aspirated, and suspensions of 100 µg of LPS-E 323 in 0 4 ml saline were injected into each chamber Three h thereafter the formed exudate was aspirated and immediately chilled to 0° C The pooled exudate was centrifuged (28 000 × g for 20 min) at 4 C in a Servall RC 2 centrifuge (Sorvall Inc. Norwalk, Conn USA) and 0 1 per cent merthiolat (w/v) was added to the supernatant The supernatant was thereafter placed in a water bath at 56 C for 30 min before being frozen at −25 C and stored until used The non-stimulated exudate was treated in the same way The exudates were checked for contaminating microorganisms by microscopy of Gram stained preparations and from anaerobic and aerobic incubation on blood agar plates

Gel Filtration

The LPS-induced inflammatory exudate was filtered on Sephadex® gels (Pharmacia Fine Chemicals AB Uppsala, Sweden) at room temperature A Sephadex G-200 column (2 5 × 43 cm) was equilibrated and eluted with phosphate − (0 02 M) buffered (pH 7 2) isotonic saline (PBS) containing sodium azide (0 02 per cent w/v) The flow rate was 4 5 ml/h, and fractions of 3 37 ml were collected Another Sephadex column of G 75 gel (2 5 × 59 cm) was stabilized and eluted with the same buffer at a flow rate of 5 25 ml/h Fractions of 3 95 ml were collected The Sephadex G 75 column was also prepared and eluted with 8 M urea in PBS Optical density (OD) was recorded at 280 nm The columns were calibrated with bovine serum albumin (BSA) with a molecular weight (MW) of 67 000 cytochrome C (MW 12 400) myoglobin (MW 17 000) (Koch-Light Laboratories Ltd Colnbrook, Bucks England) and insulin (MW 6 000) (Sigma Chemical Company St Louis, Mo USA) Non-stimulated exudate aspirated before injection of LPS into the chambers was used as controls The MW of the peaks was calculated according to Andrews (1), using the K_{av} values of the collected fractions exerting the highest chemotaxis.

Dialysis

Dialysis of the collected fractions was performed in tubings with a molecular cut-off of 3,500 (Arthur H Thomas Co., Philadelphia Pa USA). After dialysis against step-wise diluted eluent buffer in distilled water at 4 C for 24 h, the fractions were lyophilized

Equilibrium Centrifugation

Suspensions of the major chemotactic peak eluted on G 75 columns and termed the LPS-induced PMN chemotactic factor (LPS-CF) were made in the same buffer as used in Sephadex gel chromatography and adjusted to an optical density of 0 5 at 280 nm The approximate MW was then determined from data of equilibrium centrifugation using a Centriscan ultracentrifuge (MSE Centriscan 75)

Analytical Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in glass tubes (0.5 × 7 5 cm) in a Shandon apparatus (Shandon Scientific Company Ltd London) The separating gel contained 12 5 per cent (w/v) acrylamide (British Drug Houses, Ltd Poole England) The same buffer (0.37 M tris-glycine, pH 9 5) was used both in the gels and in the electrode vessels (5) When sodium dodecyl sulphate (SDS) was used this was at a concentration of 0 1 per cent (w/v) both in the gels and in the reservoirs. Samples were dialyzed overnight at room temperature against a buffer containing 70 ml tris-glycine buffer 10 ml concentrated glycerol and 1 g of SDS, made up to 100 ml with distilled water and to which 0 002 per cent bromphenol blue had been added The samples were placed in a water bath at 100° C for 1−2 min. Samples of 70µl containing approximately 100 µg of the *freeze-dried LPS-CF* was *mixed with 10 µl* of 40 per cent sucrose in distilled water and layered on top of the gels Stacking was done using a constant voltage of 20 V which was then increased to 100 V until the tracking dye had reached the lower end of the tube The gels were stained for 1 h in a solution of 0 2 per cent (w/v) Coomassie brilliant blue (R250) in 50 per cent methanol in water to which 7 per cent of glacial acetic acid was added shortly before use De-staining was performed in a solution containing 4 per cent methanol and 7 per cent acetic acid The following protein markers were used for approximate MW determinations and run electrophoretically cytochrome C (Koch-Light) and human IgG (AB Kabi, Stockholm) which was reduced with 5 per cent 2 mercaptoethanol in a water bath at 100° C for 2 min

Protein Determination

The protein content of the LPS-CF was estimated by the Folin-Ciocalteu phenol method, according to *Lowry et al* (11), using BSA (Armour Pharmaceutical Company Ltd Eastbourne, England) as the standard

Gas Liquid Chromatography

Amino acid analysis Samples of 1 mg of the pooled, dialyzed and lyophilized LPS-CF were hydrolysed in 6 N HCl at 105° C for 18 h and prepared for analysis by gas liquid chromatography according to *Roach & Gehrke* (14)

Analysis of aldoses and hexosamines The preparing of samples of the LPS-CF for the detection of aldoses and hexosamines, as well as the analysis of the samples, was peformed as described previously (20)

Rabbit Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMNs) were harvested from the peritoneal cavity of New Zealand White rabbits 8–10 h after local injection of 200 ml of a solution containing 0 1 per cent glycogen (E Merck AG Darmstadt, W-Germany) in sterile saline Garamycin® and fungizone® were added in amounts of 50 and 2 5 μg per ml of the glycogen solution, respectively The harvesting and the isolation of the cells was performed as described earlier (21) After the isolation, the PMNs were resuspended in Gey's medium containing antibiotics in amounts equal to those in the glycogen solution The Gey's medium was then adjusted to 2 per cent (w/v) solution with BSA (Armour Pharmaceutical Co Ltd), one ml of the PMN suspension containing approximately 10^7 cells

Measurement of Chemotaxis

The migration of PMNs was determined by the Boyden Micropore technique (2) using a modified Boyden chamber (19) (Neuroprobe, Bethesda, Md USA) The upper compartment of the chamber received 4×10^4 cells These were centrifuged down on a Millipore membrane (Neuroprobe) with a pore size of 3 μ before placing the membrane in the chamber (21) The lower compartment contained the test medium The chambers were incubated at 37° C in humid air for 3 h before the filters were removed, fixed, stained and mounted as previously stated (21), the cells on the upper surface of the filters were carefully sucked off Five fields of the lowermost surface of the filters were selected for counting at a magnification of X320 with the aid of a micrognd (Leitz Wetzlar 10 × 10 mm)

EXPERIMENTS AND RESULTS

In preliminary experiments inflammatory exudates aspirated from the chambers at different time intervals after LPS injection, were fractionated on Sephadex G-200 columns and the eluate tested for chemotactic activity in modified Boyden chambers. In these experiments the highest activity was found in the eluate after fractionation of exudate aspirated 3 h after local injection of LPS This exudate was, therefore, gel filtered in order to isolate the LPS-induced PMN chemotactic factor

Aliquots of 4 ml exudate were passed through G-200 columns. The major chemotactic peak was always found in the 16,000 MW region (K_{av} 0 71), slightly ahead of the cytochrome C marker (Fig. 1). Slighter but still significant chemotactic activity was retained in the eluate with peaks corresponding to K_{av} 0 41 (MW 68 000) and K_{av} 0 92 (MW 7,000). Invariably a fourth peak with K_{av} of 0 08 indicative of a MW of 300,000 was found. No significant activity could be found in the void volume or in the eluate of non-stimulated exudate.

Collected fractions of the major peak corresponding to a MW range of 12,000 to 20 000 were dialyzed, lyophilized and pooled. This pooled sample was reconstituted with eluent buffer to a volume of 0.5 ml and applied to the Sephadex G-75 column. In these experiments, three peaks of chemotactic activity were constantly found in the eluate (Fig. 1). These peaks showed the same pattern as those found in the G-200 eluate with respect to MW and mutual chemotactic activity The major peak corresponding to a MW of 16 000 (K_{av} 0.37) and that of the two other peaks were calculated to 7 000 (K_{av} 0 56) and 68 000 (K_{av} 0 05) No activity could be detected in the void volume. There was no significant change in the chemotactic activity of the fractions before and after dialysis

In another experiment, the collected pooled fractions of the major chemotactic peak eluted on G-200 columns were re-passed over a Sephadex G 75 column, equilibrated and eluted with 8 M urea in PBS After dialysis of the collected fractions they were examined for chemotactic capacity on rabbit peritoneal PMNs No activity was found either in the void volume or in the MW region corresponding to 68 000 Furthermore, the chemotactic peak with a MW of 7,000 now exhibited almost the same chemotactic activity as the peak eluted slightly behind the myoglobin marker (Fig 2). For characterization and further study of the chemotactic factor only the central fractions of the major peak eluted without urea on Sephadex G-75 columns were used

The MW of the major chemotactic peak (LPS-CF) in ten G-75 runs calculated from proteins of known molecular weights was 16 000 with a standard deviation of 470 In equilibrium centrifugation the MW of the LPS-CF calculated from two runs was 16,000 In polyacrylamide gel electrophoresis, a single band was seen after protein staining. In SDS-polyacrylamide gel electrophoresis the LPS-CF moved between the cytochrome C and the light IgG chain, and its MW was determined to be 16 400 when the moving front of the protein bands were used for calculation (Fig. 3)

The results of the quantitative amino acid analysis are summarized in Table 1 Hexosamines or neutral sugars were not detected. The mean

teristics of the PMN chemotactic factor present in the inflammatory exudate after injection of *Bactero ides fragilis* LPS into wound chambers implanted in rabbits.

MATERIALS AND METHODS

Source and Preparation of LPS

LPS (endotoxin) was isolated from *Bacteroides fragilis* subspecies *fragilis* strain Lille E 323 by phenol water extraction (27) Further purification of the LPS by ultracentrifugation and treatment of the pellet with ribonuclease and deoxyribonuclease has been reported previously (20). Stock suspensions of LPS containing 2 5 mg per ml was made in sterile isotonic saline This was treated ultrasonically (MSE/Mullard 60W 20 kc/s) at 0° C for 2 min

Inflammatory Exudate

Five Teflon® chambers (12) were implanted subcuta neously on each lateral side of the abdomen of six months-old New Zealand White rabbits as described before (24) Six days after implantation the exudate was aspirated and suspensions of 100 µg of LPS-E 323 in 0 4 ml saline were injected into each chamber Three h thereafter the formed exudate was aspirated and immediately chilled to 0° C The pooled exudate was centrifuged (28 000 × g for 20 min) at 4 C in a Servall RC 2 centrifuge (Sorvall Inc. Norwalk, Conn. USA) and 0 1 per cent merthiolat (w/v) was added to the supernatant The supernatant was thereafter placed in a water-bath at 56 C for 30 min before being frozen at − 25 C and stored until used. The non-stimulated exudate was treated in the same way The exudates were checked for contaminating microorganisms by microscopy of Gram-stained preparations and from anaerobic and aerobic incubation on blood agar plates.

Gel Filtration

The LPS-induced inflammatory exudate was filtered on Sephadex® gels (Pharmacia Fine Chemicals AB Uppsala, Sweden) at room temperature A Sephadex G-200 column (2 5 × 43 cm) was equilibrated and eluted with phosphate − (0 02 M) buffered (pH 7 ?) isotonic saline (PBS) containing sodium azide (0 0? per cent w/v) The flow rate was 4 5 ml/h and fractions of 3 37 ml were collected Another Sephadex column of G 75 gel (2 5 × 59 cm) was stabilized and eluted with the same buffer at a flow rate of 5 25 ml/h Fractions of 3 95 ml were collected The Sephadex G 75 column was also prepared and eluted with 8 M urea in PBS Optical density (OD) was recorded at 280 nm The columns were calibrated with bovine serum albumin (BSA) with a molecular weight (MW) of 67 000 cytochrome C (MW 12 400) myoglobin (MW 17 000) (Koch-Light Labora tories Ltd Colnbrook, Bucks England) and insulin (MW 6 000) (Sigma Chemical Company St Louis, Mo USA) Non-stimulated exudate aspirated before injection of LPS into the chambers was used as controls The MW of the peaks was calculated according to Andrews (1) using the K_{av} values of the collected fractions exerting the highest chemotaxis.

Dialysis

Dialysis of the collected fractions was performed in tubings with a molecular cut-off of 3,500 (Arthur H Thomas Co Philadelphia, Pa. USA). After dialysis against step-wise diluted eluent buffer in distilled water at 4 C for 24 h the fractions were lyophilized

Equilibrium Centrifugation

Suspensions of the major chemotactic peak eluted on G 75 columns and termed the LPS-induced PMN chemotactic factor (LPS-CF) were made in the same buffer as used in Sephadex gel chromatography and adjusted to an optical density of 0 5 at 280 nm The approximate MW was then determined from data of equilibrium centrifugation using a Centriscan ultracentri fuge (MSE Centriscan 75)

Analytical Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in glass tubes (0 5 × 7 5 cm) in a Shandon apparatus (Shandon Scientific Company Ltd. London) The separating gel contained 17.5 per cent (w/v) acrylamide (British Drug Houses, Ltd. Poole, England) The same buffer (0 37 M tris glycine, pH 9 5) was used both in the gels and in the electrode vessels (5) When sodium dodecyl sulphate (SDS) was used this was at a concentration of 0 1 per cent (w/v) both in the gels and in the reservoirs Samples were dialyzed overnight at room temperature against a buffer containing 70 ml tris glycine buffer 10 ml concentrated glycerol and 1 g of SDS made up to 100 ml with distilled water and to which 0 002 per cent bromphenol blue had been added The samples were placed in a water-bath at 100° C for 1–2 min Samples of 20µl, containing approximately 10 µg of the freeze-dried LPS-CF was mixed with 10 µl of 40 per cent sucrose in distilled water and layered on top of the gels Stacking was done using a constant voltage of 20 V which was then increased to 100 V until the tracking dye had reached the lower end of the tube The gels were stained for 1 h in a solution of 0 2 per cent (w/v) Coomassie brilliant blue (R250) in 50 per cent methanol in water to which 7 per cent of glacial acetic acid was added shortly before use De-staining was performed in a solution containing 4 per cent methanol and 7 per cent acetic acid The following protein markers were used for approximate MW determinations and ran electrophoretically cytochrome C (Koch Light) and human IgG (AB Kabi Stockholm) which was reduced with 5 per cent 2 mercaptoethanol in a water bath at 100° C for 2 min

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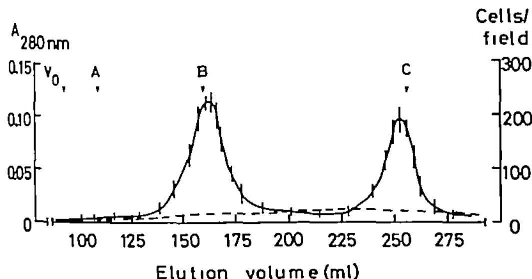


Fig 2 The elution profile of the major peak (molecular weight 16,000) of chemotactic activity (designated D in the upper panel on Fig 1), eluted on a Sephadex G-75 column with 8 M urea in phosphate-(0 02 M) buffered (pH 7.2) saline. Chemotactic activity expressed as number of cells per high-power field (solid curve). Standard deviation for triplicate chambers denoted by vertical bar. Broken curve indicates absorbance at 280 nm. V_0, void volume. Arrow heads indicate elution peak of BSA (A), myoglobin (B) and insulin (C)

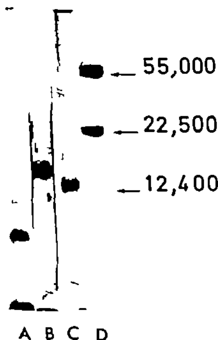
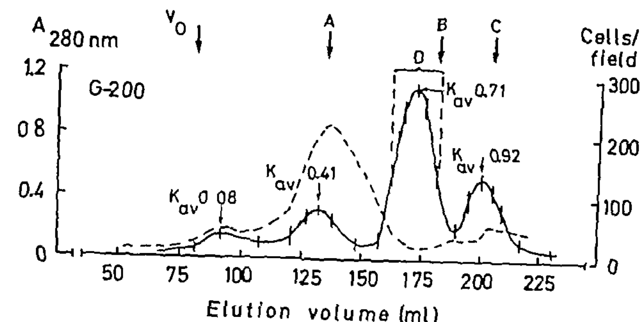


Fig 3 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of the LPS-induced PMN chemotactic factor (B), cytochrome C (C) and reduced human IgG (D). The electrophoretic pattern of the chemotactic factor is shown in the gel (A) run without SDS

TABLE 1 *A Quantitative Determination of the Amino Acid Composition of the PMN Chemotactic Factor Isolated from LPS Induced Inflammatory Exudate (pmoles/mg) Values are the Mean Obtained from two Samples*

Amino acid	pmoles/mg samples
Alanine	0.81
Valine	0.77
Glycine	3.77
Isoleucine	0.55
Leucine	1.05
Proline	1.00
Threonine	0.96
Serine	3.73
Methionine	5.19
Phenylalanine	0.57
Aspartic acid	1.56
Glutamine	3.61
Tyrosine	1.43
Lysine	0.56
Tryptophan	N D
Histidine	N.D
Arginine	N D
Cysteine	N D

N D – not determined



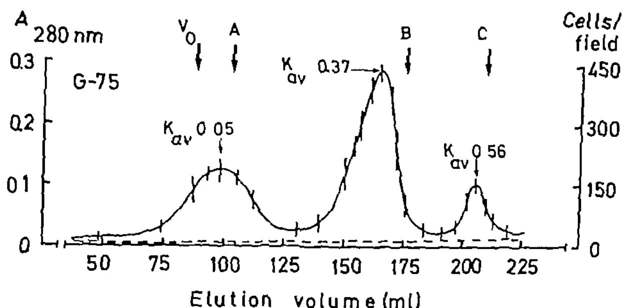


Fig 1 Upper panel the elution profile of LPS-induced inflammatory exudate on Sephadex G-200 Lower panel the elution profile of the major peak of chemotactic activity (pooled fractions designated D in the upper panel) on a Sephadex G-75 column Elution fluid phosphate-(0 02M) buffered (pH 7.2) saline. Chemotactic activity expressed as number of cells per high power field (solid curve) Vertical bar denotes standard deviation for triplicate chambers. Absorbance at 280 nm is indicated by broken curve V void volume. Arrows indicate elution peak of BSA (A), cytochrome C (B) and insulin (C)

protein content was 89 0 per cent with a standard deviation of 5 1

The activity of the LPS-induced PMN chemotactic factor when suspended in isotonic saline remained unchanged after storage for five weeks at 4° C or when frozen at −25° C and thawed at 4 C three times (Table 2). Heating at 56° C for 1 h in a water-bath did not abolish the activity of LPS-CF On the contrary a slightly increased activity was found. The activity of LPS-CF was not abolished completely before heating at 90° C for 1 h in a water-bath

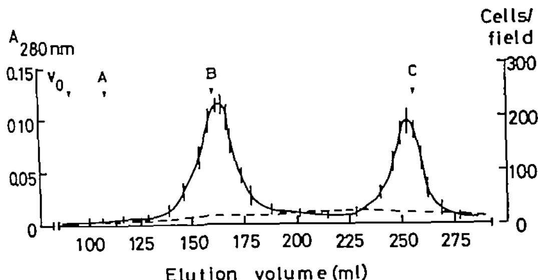


Fig 2 The elution profile of the major peak (molecular weight 16 000) of chemotactic activity (designated D in the upper panel on Fig 1), eluted on a Sephadex G-75 column with 8 M urea in phosphate-(0 02 M) buffered (pH 7.2) saline. Chemotactic activity expressed as number of cells per high-power field (solid curve). Standard deviation for triplicate chambers denoted by vertical bar. Broken curve indicates absorbance at 280 nm. V_0, void volume. Arrow heads indicate elution peak of BSA (A), myoglobin (B) and insulin (C).

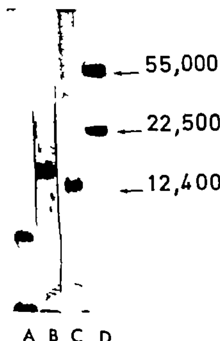


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Isoleucine	0.55
Leucine	1.05
Proline	1.00
Threonine	0.96
Serine	3.73
Methionine	5.19
Phenylalanine	0.57
Aspartic acid	1.56
Glutamine	3.61
Tyrosine	1.43
Lysine	0.56
Tryptophan	N D
Histidine	N.D
Arginine	N D
Cysteine	N D

N D – not determined

TABLE 2 *Stability of the PMN Chemotactic Factor Isolated by Gel Filtration from LPS Induced Inflammatory Exudate in Wound Chambers Implanted Subcutaneously in Rabbits*

Treatment of factor[a]	Chemotactic activity[b] Mean ± s d.
No treatment	154 ± 16
4° C for 5 weeks	152 ± 10
Frozen (–25 C) and thawed (4 C)	153 ± 20
56° C 1 h	159 ± 19
70° C 1 h	54 ± 14
80° C 1 h	48 ± 17
90° C 1 h	3 ± 4

[a] Stock suspension of 125 μg/ml sterile isotonic saline prepared and 0 2 ml tested in each Boyden chamber

[b] Migrated PMNs per high-power field calculated from triplicate filters.

DISCUSSION

The chemotactic activity of LPS-induced inflammatory exudate was examined in order to determine its relationship to previously described chemotactic mediators. The fractionation procedures showed the major chemotactic peak to be eluted at a K_{av} of 0 37 on a Sephadex G-75 column, corresponding to a molecular weight of 16 000 In this respect, the presently reported LPS-induced PMN chemotactic factor seems consistent with that reported by others when fractionating serum was pretreated with LPS (19) or with immune complexes (17) Besides, this chemotactic factor also seems to be consistent with that isolated from LPS-induced intraperitoneal exudate of guinea pigs (18)

In repeated chromatographic separations of exudate on Sephadex G-200 columns, the peak of activity with a MW of 300 000 was not always found This peak accounted for only about 15 per cent of the activity present in the major peak. This high molecular weight peak might be in accordance with that of the chemotactic trimolecular complex C5 6 7 described by *Ward et al.* (26)

The peak of chemotactic activity eluted just ahead of the BSA marker was not found when eluted with 8 M urea in PBS This interesting finding indicates that the peak with a MW of 68 000 when eluted without urea on G 200 and G-75 was an aggregation product. Its chemotactic material may possibly be made up of four units of the chemotactic material present in the peak with a MW of 16 000 Thus, this peak of chemotactic capacity might not be a leukotactic mediator independent of the main chemotactic factor as has been suggested by others (19).

The peak of activity representing MW 16 000 was found to contain the majority of the chemotactic capacity eluted The MW of the present cytotaxin thus fits well with that of a chemotactic factor of MW 15 000 generated in guinea pig serum upon treatment with LPS which has been described as a split product of C5 (19) Besides being highly chemotactic, the cytotaxin C5a also possesses anaphylatoxic activity (16). It has been proposed that this property of C5a is comparable with that of the classical anaphylatoxin (6 9). If this is so this cleavage product of C5 indeed should be considered an important mediator of the inflammatory process.

Recently *Wissler* (28) isolated two different peptides, viz. classical anaphylatoxin and cocytotaxin, from rat serum treated with immune complexes Chemotactic activity for PMNs was found to be a major property of this anaphylatoxin-cocytotaxin-leukotaxis (ACL)-system (29). Thus the anaphylatoxin peptide is active alone, whereas neither the anaphylatoxin, nor the cocytotaxin has chemotactic activity However upon recombination of the two peptides this complex is highly chemotactic. The relationship between this anaphylatoxin and C5a is questionable, but it seems likely that they are identical.

The chemotactic activity of the peak with MW 7 000 was also repeatedly found after all fractionation procedures, and the activity approached that of the major chemotactic peak with a MW of 16 000 when eluted with urea. This might indicate that the active material eluted in the MW region of 7 000 could be comprised of cocytotaxin and anaphylatoxin in a dissociated state.

The SDS-polyacrylamide gel electrophoresis indicated that the peak of MW of 16 000 was a homogeneous peptide. An apparently pure preparation then seemed to be obtained by deliberately discarding marginal fractions of the peak of MW 16 000 in the chromatographic separations. The concentration of SDS (0 1 per cent) used, may thus counteract the aggregation but does not promote a dissociation of the chemotactic principle. The estimated MW of the LPS-CF in Sephadex G-75 runs agrees with that calculated from equilibrium centrifugation.

LPS-CF was found to be rather resistant to physical treatments, and its activity was not destroyed before heating in a water-bath at 90° C for 1 h (cf Table 2) In addition, this cytotaxin was non-dialyzable as were those isolated from strangulation fluids of rats (25), and those generated in rabbit serum by immune complexes (8).

The results of this study indicate that the PMN chemotactic factor isolated from LPS-induced inflammatory exudate in rabbits is identical in nature

nd activity to that isolated from guinea pig serum upon interaction with LPS (17). This implies that C5 is the parent molecule from which the active fragment is split off.

Furthermore, this finding supports the assumption that the peak with a MW of 7 000 consists of the C5a in a dissociated state, *viz.* the classical anaphylatoxin and the cocytotaxin peptides and that this dissociated state of the C5a may also be increased when urea is used in the elution fluid. A re-association of the peptides present in the low molecular weight peak possibly occurs during dialysis which restores the chemotactic activity According to the findings reported by *Wissler et al* (29), this would imply that chemotaxis is not mediated by one single peptide, but by an interplay of peptides

The equilibrium centrifugation was kindly performed by Mr *John Berge* Dept of Biochemistry University of Bergen

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Acta path. microbiol. scand. Sect. B, 86: 237–245, 1978

RABBIT POLYMORPHONUCLEAR LEUKOCYTE CHEMOTACTIC FACTOR GENERATED *IN VIVO* BY *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE

II. Antigenic and Biologic Properties

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Sveen, K. Rabbit polymorphonuclear leukocyte chemotactic factor generated *in vivo* by *Bacteroides fragilis* lipopolysaccharide. II. Antigenic and biologic properties. Acta path. microbiol. scand. Sect. B, 86: 237–245, 1978.

Preparations of the polymorphonuclear leukocyte (PMN) chemotactic factor isolated from lipopolysaccharide (LPS)-induced inflammatory exudate in rabbits were immunogenic in guinea pigs. Complete fusion of the precipitation lines produced against anti-CF by LPS-CF (molecular weight 16,000) and material eluted on Sephadex G-200 columns with molecular weights (MW) of 68,000, 16,000 and 7,000, was found. Also, the chemotactically active material with MW of 68,000 and 7,000 eluted on G-75 columns after fractionation of the fraction of MW 16,000 from the G-200 eluate was antigenically identical to LPS-CF in double diffusion in agar. Normal rabbit serum (NRS) incubated with LPS, LPS-induced wound chamber exudate and NRS alone gave lines of precipitation against the anti-LPS-CF sera identical to that of LPS-CF. The capacity of LPS-CF to attract PMNs was significantly higher than that of LPS, and a peak in the number of PMNs in the exudate of wound chambers implanted in rabbits was found 4 h after the local injection of LPS-CF. When injected intraperitoneally in C5 deficient mice, LPS-CF stimulated a PMN migration which was only slightly below that in C5 normal mice. Antisera to LPS-CF inhibited the chemotactic activity of LPS-CF as well as that of LPS-NRS when the supernatants were tested using the Boyden's technique. Also, preincubation of PMNs with LPS-CF suppressed the migration towards a chemotactic gradient of LPS-CF molecules of these PMNs.

Key words: *B. fragilis*, lipopolysaccharide, exudate, chemotactic factor, leukocyte chemotaxis, C deficient mice.

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The cell wall lipopolysaccharide (LPS) of *Bacteroides fragilis* produces primary skin inflammation and local and generalized Shwartzman reactions in rabbits (12). It is lethal to mice and chick embryos, is pyrogenic and gelates *Limulus* amoebocyte lysate (18). Furthermore, a chemotactic activity for rabbit polymorphonuclear leukocytes (PMNs) is elaborated *in vitro* upon interaction of LPS (endotoxin) with normal guinea pig serum (13).

Recent experiments showed that when injected into Teflon® chambers implanted in rabbits and mice, *B. fragilis* LPS evoked migration of PMNs into the chambers (14, 15). The number of PMNs in the chamber exudate was dependent on the quantity of LPS injected up to a certain amount. Independent of the amount of LPS injected, a maximal cell accumulation was found 5 h after stimulation with LPS (17). In mice genetically deficient of the fifth complement factor (C5), a delayed and insignificant

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RABBIT POLYMORPHONUCLEAR LEUKOCYTE CHEMOTACTIC FACTOR GENERATED *IN VIVO* BY *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE

II Antigenic and Biologic Properties

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Sveen, K. Rabbit polymorphonuclear leukocyte chemotactic factor generated *in vivo* by *Bacteroides fragilis* lipopolysaccharide II Antigenic and biologic properties Acta path. microbiol scand Sect. B, 86 237–245 1978

Preparations of the polymorphonuclear leukocyte (PMN) chemotactic factor isolated from lipopolysaccharide (LPS)-induced inflammatory exudate in rabbits were immunogenic in guinea pigs Complete fusion of the precipitation lines produced against anti-CF by LPS-CF (molecular weight 16,000) and material eluted on Sephadex G-200 columns with molecular weights (MW) of 68,000 16,000 and 7,000, was found Also, the chemotactically active material with MW of 68,000 and 7 000 eluted on G-75 columns after fractionation of the fraction of MW 16,000 from the G-200 eluate was antigenically identical to LPS-CF in double diffusion in agar Normal rabbit serum (NRS) incubated with LPS, LPS-induced wound chamber exudate and NRS alone gave lines of precipitation against the anti-LPS-CF sera identical to that of LPS-CF The capacity of LPS-CF to attract PMNs was significantly higher than that of LPS, and a peak in the number of PMNs in the exudate of wound chambers implanted in rabbits was found 4 h after the local injection of LPS-CF When injected intraperitoneally in C5 deficient mice, LPS-CF stimulated a PMN migration which was only slightly below that in C5 normal mice Antisera to LPS-CF inhibited the chemotactic activity of LPS-CF as well as that of LPS-NRS when the supernatants were tested using the Boyden's technique Also, preincubation of PMNs with LPS-CF suppressed the migration towards a chemotactic gradient of LPS-CF molecules of these PMNs

Key words *B fragilis*, lipopolysaccharide, exudate, chemotactic factor leukocyte chemotaxis, C deficient mice

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number of cells accumulated (15) No chemotaxis was generated *in vitro* upon incubation of LPS with C5 deficient serum whereas a normal chemotaxis was induced when serum from guinea pigs genetically deficient in C4 was used (15) These results suggest that *B fragilis* LPS induce generation of a chemotactic factor via the alternative complement pathway and that C5 is the key substrate.

By Sephadex gel filtration of the exudate induced by LPS from *B fragilis* three peaks with chemotactic activity was obtained (16). The major chemotactic peak was due to a non-dialyzable peptide with a molecular weight (MW) of 16 000 One minor peak with chemotactic activity supposedly was due to aggregated material with a MW of 68 000 and another small peak with chemotactic activity was due to a component with MW of 7 000 The present paper deals with the biological activity of the chemotactically active peptide, and its antigenic relationship to the aggregated material and to the low molecular weight component.

MATERIALS AND METHODS

Isolation of the LPS Induced PMN Chemotactic Factor

The isolation of LPS from *Bacteroides fragilis* subspecies *fragilis*, strain Lille E 323 (12), the implantation in rabbits of Teflon® chambers (17) and the induction of inflammatory exudate and chemotactic factor generation by LPS (16) were carried out as earlier described The fractionation of the LPS-induced inflammatory exudate by gel filtration on a Sephadex G 200 column (Pharmacia Fine Chemicals AB Uppsala, Sweden) and further filtration of the major peak of chemotactic activity having a molecular weight (MW) of 16 000 on a G 75 column, have been described in detail in a preceding paper (16). The centrally collected fractions of the major peak of chemotactic activity (MW = 16 000) eluted on the G-75 column were lyophilized and termed LPS-CF (16) Analysis of LPS-CF in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis showed only one band after protein staining (16).

Collection of Serum

Blood from rabbits was obtained by cardiac puncture and the serum isolated by clotting of the blood at room temperature for 1 h and then at 4°C overnight The serum was pipetted off and centrifuged (IEC Universal Model UV) at 3 000 × g for 10 min, and kept in aliquots of 1 ml at −25°C until required

Production of Antiserum

Antiserum to LPS-CF was raised in guinea pigs (400–600 g) by the injection into the hind foot pads of 0 1 ml of complete Freund's adjuvant (Difco Detroit, Mich USA) and 200 μg of LPS-CF in 0 1 ml of sterile isotonic saline At days 7 and 14 after injection, equal volumes of antigen (200 μg in 0 1 ml of saline) and incomplete Freund's adjuvant (0 1 ml) were given intramuscularly at the level of the groin. Three weeks after the first injection the animals were bled by cardiac puncture. The serum was isolated as described above, decomplemented in a water-bath at 56°C for 30 min and stored in aliquots of 1 ml at −25°C until used.

Double Diffusion in Agar

Double diffusion tests were carried out in 1 per cent agar gel (Noble Agar Difco) in distilled water Undiluted antiserum and test materials were placed in wells with diameters of 3 mm at a distance of 7 mm. Following incubation for 18 h in a moist chamber at room temperature, the precipitation lines were recorded and photographed before or after staining with 0 1 per cent Amido Black 10 B The substances tested in double diffusion in agar were prepared as follows the central fractions of each peak of chemotactic activity in the eluate from Sephadex G-75 columns, were pooled and lyophilized. Test suspensions of each of these peaks were prepared in saline at a concentration of 1 mg per ml PMN chemotactic factor was generated by the incubation of endotoxin in normal rabbit serum (250 μg/ml) at 37°C for 30 min, following 56°C for 30 min After centrifugation of the incubated mixture at 28 000 × g for 20 min at 4°C in a Servall RC 2 centrifuge (Sorvall Inc., Norwalk, Conn. USA) the supernatant was pipetted off and tested. LPS-induced inflammatory exudate for testing in double diffusion was raised in in vivo chambers implanted in rabbits and was handled as described before (16). NRS was incubated at 37°C for 30 min and thereafter at 56°C for 30 min before being tested.

Immunoelectrophoresis

This was performed using an LKB apparatus (LKB Produkter Stockholm) One per cent agar gel (Noble Agar Difco) in Veronal buffer (ionic strength 0 037) was employed and electrophoresis was run for about 80 min with Veronal buffer of pH 8 6 and ionic strength 0.050 with a constant voltage of 6 to 8 V/cm

Immune Precipitates

A stock suspension of LPS in Gey's medium (1 mg/ml) was treated ultrasonically (MSE/Mullard 60W 20 kc/s) at 0°C for 1 min. Serial dilution (200 to 3 12 μg) in 0 15 ml (Gey's medium) were made and the same volume of NRS added to each After incubation at 37°C in a water-bath for 30 min and decomplementation at 56°C for 30 min, the mixtures were centrifuged (28 000 × g for 70 min at 4°C) The supernatants were pipetted off and 0 15 ml anti LPS-CF serum, which was diluted 1 to 2 in isotonic saline, was added to each dilution (6) Immune precipitates were also prepared by mixing 0 15 ml anti-LPS-CF serum to the same volume of Gey's balanced salt solution (Gey's medium), containing varying amounts of LPS-CF (stock suspension of LPS CF in Gey's medium 1 mg/ml) The concentration of LPS-CF in each dilution step differed with 10 μg and dilutions ranging from 10 to 100μg were made Precipitates were allowed to form by placing all of the reaction mixtures in a water-bath at 37°C for 2 h and thereafter at 4°C for 48 h The mixtures were centrifuged

(3 000 × g for 20 min at 4°C), and the precipitates washed twice in ice-cooled saline and resuspended in 0.1 N NaOH. The optical density of the precipitates were measured at 280 nm and the point of maximum precipitation determined. The supernatants of the various mixtures were further prepared and tested for chemotactic activity as described in Experiments and Results.

Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PNMs) were obtained from the peritoneal cavity of New Zealand White rabbits 4-10 h after intraperitoneal (i.p.) injection of 200 ml of a solution containing 0.1 per cent glycogen (E. Merck AG, Darmstadt, W-Germany) in sterile isotonic saline. Garamycin® (Schering Corporation, Bloomfield, N.J., USA) and fungizone® (Squibb Flow Laboratories, Irvine, Scotland) were added in amounts of 50 and 2.5 µg per ml solution, respectively. The PMNs were harvested and isolated from the exudate as described before (13). The PMNs were resuspended in Gey's medium containing antibiotics and bovine serum albumin (Armour Pharmaceutical Company Ltd., Eastbourne, England) (2 per cent w/v) and adjusted to a concentration of 10^7 cells per ml. More than 93 per cent of the cells resisted staining with trypan blue.

Measurement of Chemotactic Activity

In vitro chemotaxis was examined with the Boyden Millipore technique (1), using a modified Boyden chamber (11) (Neuroprobe, Bethesda, Md., USA). The two compartments of the chamber were separated with a Millipore filter (Millipore Filter Corp., Bedford, Mass., USA) with a pore size of 3 µ. The upper compartment received 4 × 10^6 cells which were centrifuged down on the filter before this was placed into the chamber (13). The test media were placed in the lower compartment and were prepared as described in Experiments and Results. After incubation of the chambers in humid air at 37°C for 3 h, the cells on the upper surface of the filters were gently sucked off. The filters were fixed, stained and mounted as previously described (13). For calculation of the chemotactic activity five high-power fields of the lowermost surface of the filters were selected and the cells counted under light microscopy at a magnification of X 320, using a micrograd (Leitz Wetzlar 10 × 10 mm) (13).

PMN Preincubation in Chemotactic Medium

Rabbit peritoneal PMNs were incubated at 37°C for 30 min with LPS-CF at various concentrations in Gey's medium. PMNs in Gey's medium only were incubated and used as the control. After washing the PMNs three times with ice-cooled Gey's medium, they were stimulated for migration against a chemotactic gradient consisting of 50 µg/ml of LPS-CF in Gey's medium in the modified Boyden chamber.

Measurement of Leukocyte Migration into Wound Chambers

Teflon® chambers were implanted subcutaneously in six-months-old New Zealand White rabbits, five chambers on each lateral side of the abdomen as described earlier (17). The exudate formed six days after implantation was aspirated and immediately chilled to 0°C and replaced by a suspension of 25 µg of LPS-CF or 25 µg of LPS in 0.4 ml of isotonic saline. Saline alone was used as control. The amounts of exudate (wound fluid) collected from each chamber before and 5 h after injection of test suspension or saline, were measured and the number of leukocytes per chamber was calculated as before (14).

Kinetics of Leukocyte Migration into Wound Chambers

At different time intervals after injection of 25 µg of LPS-CF in 0.4 ml of sterile isotonic saline into the wound chambers, exudate was withdrawn from successive chambers and the number of leukocytes per µl of exudate calculated as before (17). The relative percentage of PMNs and mononuclear leukocytes (MNs) in the exudate was calculated as previously (17). The exudate aspirated six days after the implantation of the chambers was examined for contaminating microorganisms by microscopy of Gram-stained preparations. Aerobic and anaerobic incubation on blood agar plates was also performed.

Measurement of Migration of Leukocytes into the Peritoneal Cavity

Migration of PMNs into the peritoneal cavity was tested by injection of 25 µg of LPS-CF in 0.9 ml of isotonic saline and of 25µg of LPS in 0.9 ml isotonic saline i.p. into genetically C5 deficient (C5D) mice of the strain DBA/2J weighing 28 ± 2 g and in C5 normal (C5N) mice of the strain NMRI weighing 32 ± 3 g. Mice of both sexes were used, and the strains were caged separately. The mice were killed after 5 h. The harvesting of the cells from the peritoneal cavity as well as the calculation of the total number of leukocytes present in the exudate harvested from each mouse were performed as described before (15).

The media used in the experiments were sterilized before use by passage through a filter with a pore size of 0.45 µ (Millipore Filter Corp.).

Statistical Method

Standard deviation was calculated as previously (17). For evaluation of differences in chemotactic activity the Wilcoxon rank test for two samples was used (2). Values of $p \leq 0.05$ were accepted as statistically significant.

EXPERIMENTS AND RESULTS

Injection into guinea pigs of LPS-CF elicited an antibody response with a reciprocal precipitin titre varying from 16 to 64. This antibody gave only one precipitation line in double diffusion against the antigen.

The chemotactically active materials with MW of 68 000 and 7 000 present in the eluate from Sephadex G-200 and G-75 columns, were tested in agar double diffusion against antiserum to LPS-CF. As shown in Fig. 1 the three peaks of chemotactic

number of cells accumulated (15). No chemotaxis was generated *in vitro* upon incubation of LPS with C5 deficient serum whereas a normal chemotaxis was induced when serum from guinea pigs genetically deficient in C4 was used (15). These results suggest that *B. fragilis* LPS induce generation of a chemotactic factor via the alternative complement pathway and that C5 is the key substrate.

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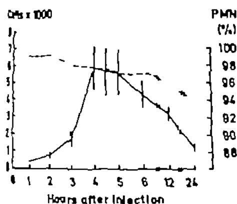


Fig 6 Kinetics of leukocyte accumulation in wound chambers implanted in rabbits after local injection of 25 µg of LPS-CF in 0.4 ml saline. The chemotactic activity is expressed as number of cells per µl of exudate. Each point represents the mean cell count ± standard deviation (vertical bar) for triplicate chambers (solid curve). The percentage of PMNs is denoted by the broken curve.

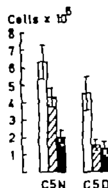


Fig 7 Induction of PMN migration into the intraperitoneal cavity of C5 normal (C5N) and C5 deficient (C5D) mice 5 h after local injection of 25 µg of LPS-CF (open columns) and from 25 µg of LPS (hatched columns). Solid columns are saline only. Each column represents the mean number of cells ± standard deviation (vertical bar) from six mice. The higher chemotactic activity of LPS-CF in C5N mice is statistically significant at the 5 per cent level as the higher activity of LPS is at the one per cent level.

TABLE I *Chemotactic Activity Induced by the Supernatant from Incubation of Anti LPS-CF Serum with Varying Amounts of LPS-CF or LPS in Normal Rabbit Serum (NRS)*

Reaction mixture	Cells/field (n = 6) ± s d
1 LPS-CF (50 µg) + anti-LPS-CF serum	0 8 ± 1.3
LPS-CF (60 µg) + anti-LPS-CF serum[b]	7 8 ± 3 9
LPS-CF (70 µg) + anti-LPS-CF serum[c]	100.1 ± 50 6
2 LPS (12 5 µg) + anti-LPS-CF serum[a]	2.0 ± 2.3
LPS (25 µg) + anti-LPS-CF serum[b]	3 6 ± 4 8
LPS (50 µg) + anti-LPS-CF serum[c]	40 5 ± 36 3
3 Controls	
LPS-CF (25 µg) + 0 6 ml Gey's medium[d]	213 3 ± 25 2
LPS (60 µg) + 0 6 ml NRS + 0 6 ml Gey's medium	207.5 ± 35 7
NRS alone	26.7 ± 4.2

Test samples were prepared as follows

1 LPS-CF in 0 15 ml Gey's medium + 0 15 ml anti-LPS-CF serum with addition of 0 9 ml Gey's medium to the supernatant.

2 LPS in Gey's medium + 0 15 ml NRS – 37°C 30 min ~ 56°C 30 min + 0 15 ml anti-LPS-CF serum with addition of 0 9 ml Gey's medium to the supernatant.

3 LPS (60 µg) + 0 6 ml NRS ~ 37°C 30 min ~ 56°C 30 min + 0 6 ml Gey's medium.

[a] Reaction mixture with antibody in excess
[b] Reaction mixture with maximum precipitation.
[c] Reaction mixture with antigen in excess
[d] The LPS-CF was tested only in one experiment
[e] Mean of two experiments

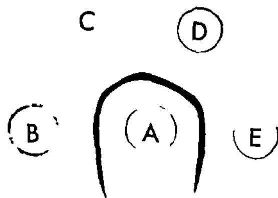


Fig 1 Immunodiffusion in agar gel Well A contains antiserum against LPS-CF Wells B C and D contain chemotactically active materials eluted on G-200 columns with molecular weights of 7 000 16 000 and 68 000 respectively Well E contains LPS-CF Stained with Amido Black 10B.

activity with MW of 68 000 16 000 and 7 000 constantly found in the G-200 eluate, gave precipitation lines against anti-LPS-CF serum, fusing completely with LPS-CF Furthermore, the chemotactic materials with MW of 68 000 and 7 000 eluted on the G-75 column, gave one precipitation line each fusing completely with that of the peak of MW 16 000 (Fig. 2). Moreover precipitation analysis in agar showed complete fusion of the lines formed by LPS-CF LPS-serum incubation mixture and LPS-induced inflammatory wound chamber exudate with antiserum to LPS-CF (Fig 3) Also when testing NRS one faint line of precipitation fusing completely with that formed by the LPS-CF

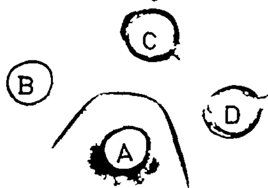


Fig 2 Immunodiffusion slide in agar gel Antigenic reaction between the chemotactic material eluted from G-75 columns with molecular weights of 7 000 (well B), 16 000 (well C) and 68 000 (well D) Antiserum (well A) was raised against the material of molecular weight 16 000 (LPS-CF)

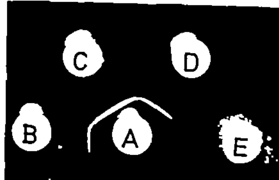


Fig 3 Double diffusion in agar gel. Antigenic reaction between antiserum raised in guinea pigs against LPS-CF (well A), LPS-NRS preincubated at 37°C for 30 min and at 56°C for 30 min (well B), LPS-CF (well C), wound chamber exudate 3 h after induction with LPS decomplemented at 56°C for 30 min (well D) and NRS activated for 37°C for 30 min and deactivated at 56°C for 30 min (well E). The faint line from well E, which is identical to the precipitation line demonstrated cannot be seen on the photograph.

was seen. In immunoelectrophoresis, LPS-CF showed a low electrophoretic mobility showing one single precipitation arc near the application well when anti LPS-CF serum was used (Fig 4).

High migration rates of PMNs into *in vivo* chambers of rabbits were induced when LPS-CF was applied into the chambers (Fig. 5). When the



Fig 4 Immunoelectrophoresis in agar gel of LPS-CF Guinea pig antiserum to LPS-CF in the trough The arrow crossing the application point indicates the *precipitation arc.*

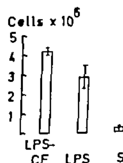


Fig 5 PMN accumulation in wound chambers in response to LPS-CF and LPS Doses of 25 µg of each preparation were tested. Saline (*S*) was used as control Each column represents the mean number of cells ± standard deviation (vertical bar) from five chambers The higher chemotactic activity induced by LPS-CF is at the one per cent level

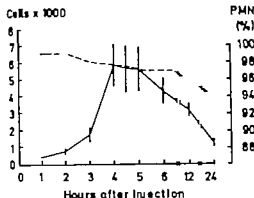


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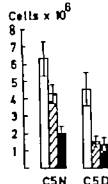


Fig 7 Induction of PMN migration into the intraperitoneal cavity of C5 normal (C5N) and C5 deficient (C5D) mice 5 h after local injection of 25 µg of LPS-CF (open columns) and from 25 µg of LPS (hatched columns). Solid columns are saline only. Each column represents the mean number of cells ± standard deviation (vertical bar) from six mice. The higher chemotactic activity of LPS-CF in C5N mice is statistically significant at the 5 per cent level as the higher activity of LPS is at the one per cent level.

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LPS-CF (60 µg) + anti-LPS-CF serum[b]	7.8 ± 3.9
LPS-CF (70 µg) + anti-LPS-CF serum[c]	100.1 ± 50.6
2 LPS (12.5 µg) + anti-LPS-CF serum[a]	2.0 ± 2.3
LPS (25 µg) + anti-LPS-CF serum[b]	3.6 ± 4.8
LPS (50 µg) + anti-LPS-CF serum[c]	40.5 ± 36.3
3 Controls	
LPS-CF (25 µg) + 0.6 ml Gey's medium[d]	213.3 ± 25.2
LPS (60 µg) + 0.6 ml NRS + 0.6 ml Gey's medium	207.5 ± 35.7
NRS alone	26.7 ± 4.2

Test samples were prepared as follows

1 LPS-CF in 0.15 ml Gey's medium + 0.15 ml anti-LPS-CF serum with addition of 0.9 ml Gey's medium to the supernatant.

2 LPS in Gey's medium + 0.15 ml NRS – 37°C 30 min – 56°C 30 min + 0.15 ml anti-LPS-CF serum with addition of 0.9 ml Gey's medium to the supernatant.

3 LPS (60 µg) + 0.6 ml NRS – 37°C 30 min – 56°C 30 min + 0.6 ml Gey's medium

[a] Reaction mixture with antibody in excess

[b] Reaction mixture with maximum precipitation

[c] Reaction mixture with antigen in excess

[d] The LPS-CF was tested only in one experiment.

[e] Mean of two experiments

chemotactic activity of equivalent amounts of LPS-CF and LPS were compared, a significantly higher migration activity of PMNs into the wound chamber fluid was found where LPS-CF had been injected 5 h before ($p < 0.01$)

Figure 6 depicts the activity of LPS-CF for rabbit PMNs plotted as a function of time. The influx of PMNs into the wound chambers was found to increase more than three times between the third and the fourth hour after the injection of LPS-CF Thereafter only a slow decrease of the PMN migration took place. More than 97 per cent of the cells present in the exudate at the peak of migration activity 4 h after injection of LPS-CF were made up of PMNs.

The application of LPS-CF intraperitoneally in C5D mice stimulated the PMNs to active migration (Fig. 7). Although the accumulation of cells in C5N mice was markedly higher than in C5D mice ($p < 0.05$) the higher mobilization rate induced by LPS-CF than by equivalent amounts of endotoxin on PMNs in C5D mice, was highly significant ($p < 0.01$). The higher number of cells accumulated i p in C5N mice than in C5D mice after LPS stimulation, was statistically significant at the one per cent level

Table 1 summarizes the results of the inhibition studies of the anti LPS-CF serum on the chemotactic capacity induced by the purified LPS-CF and that of LPS when incubated in normal rabbit serum The zone of maximum precipitation was found to correspond to 60 µg of LPS-CF and to 25 µg of LPS in the respective reaction mixtures. Significant chemotactic activity was found in the antigen excess zone whereas no chemotaxis was induced in the equivalence zone or in the antibody excess zone.

The inhibitory effect on the chemotactic response of PMNs when preincubated with varying concentrations of LPS-CF and thereafter challenged in the modified Boyden chamber is shown in Table 2 The degree of inhibition was found to be related to the amount of LPS-CF used in the preincubation

TABLE 2 *Suppression of Chemotactic Response by Preincubation of PMNs with Varying Amounts of LPS-CF and Challenged with 50 µg/ml of LPS-CF*

Preincubation of PMNs with	Inhibition of chemotaxis (%)[a]
LPS-CF 50 µg/ml	82 7 ± 4 3
LPS-CF 25 µg/ml	56 6 ± 11 1
LPS-CF 12 5 µg/ml	26 6 ± 9 4

Results represent mean and standard deviation of one experiment performed in triplicate.

DISCUSSION

As reported in a previous paper (16), chromatography of LPS-induced inflammatory exudate on Sephadex G 200 columns constantly showed three peaks with chemotactic activity The molecular weights were estimated to 68 000 16 000 and 7 000

C5 has been found to be the precursor of both the chemotactic and the anaphylatoxic activity (5 8), and it has been proposed that this anaphylatoxin peptide may be identical to classical anaphylatoxin (4 19). According to *Wissler* (22) and *Wissler et al* (23) the classical anaphylatoxin (probably C5a) was not chemotactic on its own, but when combined with another peptide (cocytotaxin) which was neither anaphylatoxic nor chemotactic on its own, the chemotactic activity was restored. The molecular weights of these peptides were reported to be about 8,500–9,500 Thus, the fraction of MW 7 000 may contain both of these peptides in a dissociated state, the component of MW 16 000 being identical to C5a and the fraction of MW 68 000 a polymer of the component of MW 16 000 Also the finding that the chemotactic material present in the three peaks was antigenically identical to that of LPS-CF (cf Fig. 1) strengthens the suggestion that the material of MW 68 000 is a polymer of the fraction of MW 16 000 Furthermore, this suggestion is supported by the finding that the filtration on Sephadex G-75 column of the major peak (MW 16 000) from the G-200 column gave three peaks which were antigenically identical to LPS-CF (cf Fig. 2) In addition, support was found for the supposition that the fraction of MW 68 000 is a polymer of the peak of MW 16 000 since this high molecular peak was not found in the eluate of G-75 columns when the peak of MW 16 000 from the G 200 column was filtered in the presence of 8 M urea (16). The present results clearly demonstrate that the three components mentioned are antigenically identical thus indicating a reaggregation of peptides of the peak of MW 7 000 as suggested in (16) Another possibility could be that guinea pigs have not responded to one of the peptides. However both peptides have been found to be necessary for the chemotactic activity (23) and consequently it is difficult to determine if the antiserum is directed against one or both peptides, although the antiserum inhibited chemotaxis

The fusion of the precipitation lines produced with anti LPS-CF serum, LPS-serum incubation mixture and LPS-induced wound chamber exudate with the precipitation line produced by isolated LPS-CF (cf Fig 3) shows the heat-stable leukotactic factor present in these test media to be antigenically identical to LPS-CF Furthermore, this finding

confirms the supposed identity between the humoral leukotactic mediator generated by endotoxin *in vivo* and that induced *in vitro* upon incubation of LPS with normal serum. The identity of these leukotactic mediators was further substantiated since anti-LPS-CF serum also inhibited the chemotactic activity from incubations of LPS with normal rabbit serum when antibody either was in the equilibrium or in the excess zone on the precipitation curve (cf. Table 1). However chemotactic activity was found in the supernatants of the precipitates where antigen was added beyond that needed at equivalence. It has been found that soluble immune complexes chemotactically active for PMNs are formed in the antigen excess zone (9). Such complexes might carry potent biological activity and may thus be quite relevant to the development of acute inflammatory processes. These immune complexes also have a long persistance in the blood due to the slow elimination by fixed macrophages (7). Whether the chemotactic activity present in the supernatant is due to unbound chemotactic mediator(s) or to soluble antigen-antibody complexes, is not known.

In a previous report (15) the generation of the PMN chemotactic factor by LPS *in vivo* or upon incubation with normal serum, was found not to be dependent on C4 but dependent on C5. This implies that an intact alternative complement pathway is required for the activation by LPS. Upon intraperitoneal injection in C5D mice, LPS-CF caused a PMN migration only slightly lower than in C5N mice ($p < 0.05$) (cf. Fig. 7). Moreover the comparatively higher chemotaxis induced by 25 µg of LPS-CF than by equivalent amounts of LPS when injected into *in vivo* chambers ($p < 0.01$) also demonstrates its potency (cf. Fig. 5). Thus, these findings, in conjunction with other (10, 11) and own (15, 16) reports, give considerable support to the concept that C5 is the parent substrate from which the PMN chemotactic factor is split off upon interaction with LPS.

The time elapsed from injection of LPS-CF to a peak of cell concentration in the wound chamber exudate was 4 h (cf. Fig. 6). This time lag is 1 h shorter than when LPS was examined (17), and may indicate how much time is needed for the key substrate to arrive into the chamber and the generation of the cytotaxin necessary for establishing a leukotactic concentration gradient. Furthermore, when LPS-CF was applied into *in vivo* chambers, a significantly greater increase in cell accumulation was achieved in the exudate compared to when the same amount of LPS was injected. This finding indicates that a larger bulk of PMNs in the vicinity of the chamber lumen is being stimulated for migration when LPS-CF is introduced into the chamber than when LPS is injected. These PMNs may primarily be mobilized from the vessels of the granulation tissue within and around the chamber (17) together with cells residing in the granulation tissue. It seems that a gradient of the generated cytotaxin is elaborated at a slower rate of speed when LPS is injected, and this delay may be due to a rather slow influx into the chambers of the substrate necessary for the generation of the leukotactic mediator.

The finding of an increase in accumulation of PMNs in the early phase of the inflammatory response to the injection of LPS-CF (cf. Fig. 6) corroborates previous results when LPS was tested (17). The bell-shaped curve of time versus cellular response thus seems to be typical for the migration of PMNs into an inflammatory focus, and this experiment gives valuable information about the rate at which the PMNs are mobilized at different time intervals after the induction of an inflammatory state. At the peak of cell concentration at 4 h, only about 3 per cent of the cells were MNs. The sequential migration of the two cell types cannot be attributed entirely to their responding to different chemotactic stimuli. An increasing amount of disintegrated cells were observed in the inflammatory exudate 5 to 6 h after stimulation with LPS (17), and this corresponds well with the half-time of 6 to 7 h of the PMN survival in the circulation. As depicted in Fig. 6 there is a significant decrease in number of PMNs in the inflammatory exudate 6 h after stimulation with LPS-CF. If the lower PMN migration is due to chemotactic factor inactivators in the exudate or due to absorbance of the chemotactic factor to cells trapped in the exudate, is not known. Neither is it known whether the delayed migration of MNs into LPS-CF induced inflammatory exudate is due to their slow motion, or whether they are being attracted by degradation products from the degenerating PMNs or from a complement-derived chemotactic mediator.

The preincubation of rabbit peritoneal PMNs with various amounts of LPS-CF was found to suppress the PMNs from migration towards the chemotactic gradient of the LPS-CF in the Boyden chamber (cf. Table 2). *Ward & Becker* (21) called this phenomenon deactivation. This is consistent with the concept that the receptor sites on the surface of the PMNs are saturated by preincubation with LPS-CF and that recognition of the chemotactic gradient in the Boyden chamber thereby is prevented. Cross-deactivation between the cytotaxins C5a and kallikrein (3) and between different complement derived chemotactic factors (20) have been reported.

In a previous report it was established that LPS when injected i.p. into C5D mice or into chambers implanted subcutaneously in C5D mice, do not bring about a migration of PMNs in the early phase of a normal inflammatory reaction (15). This study however shows that the PMN chemotactic factor isolated from LPS-induced inflammatory exudate of tissue chambers in rabbits activate the PMNs in C5D mice to migrate towards a gradient of chemotactic molecules in the peritoneal cavity. In addition, the only slightly lower activity exerted by LPS-CF in C5D mice compared to that in C5N mice (cf. Fig. 7), shows that the PMNs of the C5D mice respond normally to leukotactic mediators and that the fifth complement factor is crucial in a normal inflammatory response.

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BRIEF REPORT

VIBRIO PARAHAEMOLYTICUS ISOLATED FROM DISCHARGE FROM THE EAR IN TWO PATIENTS EXPOSED TO SEA WATER

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Olsen, H. *Vibrio parahaemolyticus* isolated from discharge from the ear in two patients exposed to sea water. Acta path. microbiol. scand. Sect. B, 86: 247–248, 1978.

Vibrio parahaemolyticus and *Vibrio alginolyticus* were isolated from discharge from the ear in two patients exposed to sea water. Both had perforation of the tympanic membrane. They recovered quickly. The bacteriological characteristics of the two strains are described.

Key words: *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, isolation, discharge from the ear.

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The halophilic marine vibrio, *Vibrio parahaemolyticus* biotype 1, is well established as a cause of human gastroenteritis in Japan (4, 10), but cases have also been reported in the United States (3). The organism has been found in sea water in the continental United States, Japan, Hawaii and the Far East (1, 7, 8, 9), but also in the North Sea and Baltic Sea (5).

Vibrio alginolyticus, *Vibrio parahaemolyticus* biotype 2, is another sea water vibrio closely related to the above-mentioned. It has been isolated from septic lesions of the skin in patients exposed to marine environment (6).

Case reports

Case 1. A 7-year-old boy had serous, relapsing otitis media treated with drainage of the middle ear. He was bathing in the sea when perforation of the tympanic membrane was still present. There was discharge from the ear, and a swab yielded numerous colonies of *Vibrio parahaemolyticus* in pure culture. The lesion healed spontaneously.

Case 2. While playing during sea bathing a 19-year-old man got a rupture of the tympanic membrane. Discharge from the ear followed, and a swab gave profuse growth of *Vibrio alginolyticus* in pure culture. The lesion responded to conventional treatment, including local application of terramycin.

Results

Growth after overnight incubation appeared on blood agar plates, Conradi Drigalski plates, and agar plates containing 7.5% NaCl.

Growth of the strain from case 1 was scanty on the salt plate. No haemolytic activity was demonstrated either on horse blood agar plates or on human blood agar plates. The strain from case 2 swarmed on agar plates and on horse and human blood agar plates, while the strain from case 1 swarmed only on human blood agar plates and only slightly. In semisolid agar, growth occurred at 22°, 30° and 35° C. Reduced growth was found at 10° C and 42° C and there was no growth at 4° C. Stained films showed a pleomorphic gram negative organism. The cells were highly motile by a single polar flagellum.

The following biochemical tests were positive: oxidase, catalase, gelatinase, indole production, nitrate to nitrite and lysine decarboxylase. The following were negative: urease, phenylalanine deaminase, arginine dihydrolase, ONPG, malonate utilization and hydrogen sulphide production.

Acid was produced within two days from glucose, laevulose, galactose, maltose, trehalose, mannitol, glycerol and starch. No acid was formed in 30 days from xylose, rhamnose, lactose, raffinose, adonitol, dulcitol, sorbitol, inositol, erythritol, sorbose and salicin. No gas was produced from glucose, mannitol and inositol.

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BRIEF REPORT

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TABLE 1 *Tests Showing Differences between the Strains Isolated from the Two Cases*

	Strain from	
	Case 1	Case 2
Voges Proskauer	−	+
Acid from sucrose	−	+
Acid from arabinose	+	−
Ornithine decarboxylase	+	−
Methyl red	+	−
Citrate utilization	+	−

There was sensitivity to sulphonamides, streptomycin, gentamicin, tobramycin, chloramphenicol, tetracycline, cotrimoxazole, nitrofurantoin and novobiocin and resistance to penicillin ampicillin, carbenicillin and fusidic acid.

The tests showing differences between the two strains are shown in Table 1

Discussion

The strain isolated from case 1 has the same characteristics as the Japanese strains isolated from patients with gastroenteritis, except that β-galactosidase, consistently produced by the Japanese was not demonstrated in the Danish (10). The strain from case 2 was identified as *V alginolyticus* (2 6 10)

Heamolytic activity on human blood has previously been associated with pathogenicity for man. However among strains isolated from patients with gastroenteritis, only 63% heamolyzed human erythrocytes and none equine erythrocytes (10). Sheep erythrocytes have been found to be heamolyzed frequently (7). The two strains presented here showed no heamolytic activity

The disease was mild in both patients and they recovered quickly A common feature was perforation of the tympanic membrane at the time of exposure to sea water

V parahaemolyticus has been isolated from coastal areas in several places, including Scandinavian waters However gastoenteritis caused by the microorganism is known only in Japan and the United States In Japan the consumption of sea foods is extensive and the food is often eaten raw (8), which might explain the occurrence there. The temperature has an influence on the content of *V parahaemolyticus* in sea water since the bacteria have been isolated more frequently in the summer than in the winter (1,5) This might contribute to explaining the absence of gastroenteritis in temperate geographical areas.

The bacteriological diagnosis was kindly confirmed by the *Institute of Veterinary Microbiology and Hygiene, Royal Veterinary and Agricultural University* Copenhagen, and the *Department of Diagnostic Bacteriology Statens Seruminstitut*, Copenhagen.

The writer is also grateful to colleagues who provided clinical information.

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BRIEF REPORT

THE PRESENCE IN FAECAL EXTRACTS OF BACTERIAL CELL WALL COMPONENTS RESEMBLING VIRAL STRUCTURES

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Dalen, A. The presence in faecal extracts of bacterial cell wall components resembling viral structures. Acta path microbiol scand Sect. B, 86 249–251 1978

Electron microscopy of faecal extracts frequently shows organized structures which have a superficial resemblance to viral material in that it is more heterogeneous and consists mainly of vesicles and tubules covered by subunits arranged in different patterns. Evidence is presented that this kind of material is derived from detached outer layers of bacterial cell walls

Key words Faeces, bacterial cell wall structures, viral structures

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The examination of faecal extracts by electron microscopy has become an established method in diagnostic virology. A simple preparative procedure can be used such as the one employed in our laboratory. Faeces is suspended in five volumes of saline and debris removed by centrifugation (5000 g for 30 min). One ml of the supernatant is mixed with 5 ml of phosphate buffered saline and centrifuged at 45,000 g for 1 h. The pellet is suspended in a few drops of distilled water and one drop of suspension is transferred to a carbon-formvar-coated grid (400 mesh). The fluid is removed after 60 s with a piece of filter paper and the grids are stained with 2% (w/v) sodium phospho-tungstate (PTA), pH 6.6 for 30 s. The specimens are then observed by electron microscopy (Hitachi, HU-12A).

Organized structures superficially resembling viruses or viral components are often observed in such preparations (Fig. 1). These structures are probably of bacterial origin. External layers with regularly arranged subunits have been demonstrated in many genera of both gram negative and gram positive bacteria (3). The subunits are proteins which are arranged tetragonally in gram positive bacteria and hexagonally or tetragonally in gram negative bacteria (4). In many instances detached subunits have been shown to reassemble into regular arrays with the same dimensions as in the intact bacteria (6).

Bacteria with clearly exposed structures of the superficial layers of the cell wall are sometimes found in faecal preparations (Fig. 2). The outer envelope of the bacterium in this figure is partly detached and a regular array of subunits is revealed. The surface resembles that demonstrated in freeze etched preparations of gram negative bacteria with hexagonally arranged external subunits (1). The irregularly spaced rings on the surface have an average internal diameter of 22 nm and an outer diameter of 29 nm. Similar rings but of smaller size have been observed on endotoxin blebs prepared from *Neisseria gonorrhoeae* (5).

Heat extracted subunits from the external layers of *Spirillum serpens* tend to be organized in vesicles or tubules and are probably situated on a backing layer of lipopolysaccharide (2). The subunits resemble those on debris from faecal extracts seen in Fig. 1. Such structures sometimes have the size and appearance of full or empty viral capsids.

Further purification of the heat extracted material from *Spirillum serpens*, involving removal of lipopoly saccharide, gives structures of hexagonal subunits held together by Y-shaped links (1). Sheets built up of similar cogwheelshaped subunits are sometimes found in the











The figures 1–5 are electron micrographs of faecal extracts negatively stained with 2 per cent PTA, pH 6 6 The marker on each micrograph indicates 100 nm

Fig 1 Organized structures probably derived from bacterial cell walls and consisting of hexagonal subunits situated on a backing layer X 135 000

Fig 2 Part of a rod-shaped bacterium which shows a partly ruptured outer border Note the regular arrays of hexagonal subunits and the *irregularly spaced rings of* varying sizes on the surface. X 185 000

Fig 3 A sheet of hexagonal subunits held together by Y shaped links X 240 000

Fig 4 A commonly occurring hexagonal meshwork X 240 000

Fig 5 A hexagonal meshwork in close proximity to a layer consisting of hexagonal subunits X 240 000

faecal extracts (Fig. 3). The units of which this structure is made up have a diameter of 9 nm with a centre to centre spacing of 15 to 16 nm. This type of structure is found occasionally while sheets of varying size consisting of a hexagonal meshwork are more commonly observed (Fig. 4). The length of the sides of each hexagon is 7 nm. This hexagonal meshwork is probably the basic arrangement on which the hexagonal subunits are seated, since transitional forms between the two kinds of pattern were observed occasionally (Fig. 5).

The degradation products previously obtained from bacteria by physico-chemical means are thus similar to those found in the faecal extracts of the present material. The number of these structures was found to vary considerably. In some cases the treatment of the patient with antibiotics might have contributed to the presence of bacterial debris in the faecal extracts. From a practical point of view, the presence of large numbers of such bacterial structures can interfere with the identification of particularly rotavirus when only a few viruses are present.

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Acta path. microbiol. scand. Sect. B, 86: 253–255, 1978

BRIEF REPORT

THE FIRST TICK BORNE ENCEPHALITIS VIRUS ISOLATES FROM NORWAY

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Department of Virology, National Institute of Public Health, Oslo and Institute of Medical Biology, University of Tromsø, Department of Environmental Toxicology, National Institute of Public Health, Oslo, Institute of Zoology, University of Oslo, Norway

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Key words: TBE virus; *Ixodes ricinus*; virus isolations.

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From approximately 6000 *I. ricinus* and *I. uriae* ticks collected in 1973–75, Uukuniemi (UUK) group viruses and »Runde« virus were isolated (11, 12), but no TBE related viruses were found. This paper reports the isolation and identification of TBE-group viruses from ticks collected in 1976.

Materials and Methods

Isolation Procedures

These have been described in detail elsewhere (11, 12). Ticks collected by the blanket drag method were divided into pools according to stage and sex, triturated and inoculated intracerebrally (ic) on 1–3 day-old mice. Suspensions of brains from sick or moribund mice were inoculated on new litters after having passed 220 nm filters (Millipore). Brain suspensions from the second baby mouse passage were seeded on BHK 21/c13 cell cultures in Roux bottles, which were inspected daily for cytopathic effect (CPE). Virus isolates from mouse brains and cell cultures were identified and compared by serological techniques.

Identification Procedures

A TBE virus strain (Hypr) was delivered as a 10% lyophilized baby mouse brain suspension from the 63rd passage. It underwent two additional passages in our laboratory and was used for reference antigen production. Reference antiserum/ascitic fluids were prepared in adult mice (12) after beta-propiolactone inactivation of the first immunizing inoculum (9). Antiserum was also produced in rabbits by injection of supernatants from infected HeLa cell cultures (13). These antisera were absorbed with packed HeLa cells prior to use in serological tests.

Mouse antisera and ascitic fluids to TBE, UUK (S 23), Tribec, WEE and EEE were delivered by the Yale Arbovirus Research Unit. Mouse antisera/immune ascitic fluids to the Norwegian UUK strains, Tribec and Tahyna were produced as reported previously (12).

The TBE reference virus or other arbovirus strains were not handled in our laboratories during the weeks prior to the successful isolations reported in this paper.

Antigens for serological identification were produced by sucrose-acetone (SA) extraction of second passage baby mouse brains (3).

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Results

Five virus strains related to the TBE complex were isolated from 16 tick pools comprising 358 individuals. These ticks were collected in Sogn & Fjordane County from June 8 to 11 1976. Some data concerning the isolates are shown in Table 1. The original isolations were made in October–November 1976. Reisolation was attempted twice, in December 1976 and in April 1978, both times with success.

As shown in Table 2, the serological relationship of these isolates to TBE was clear-cut by all the methods employed. The HA titres with trypsinized human and duck erythrocytes were identical (640–1280) for all strains and also for TBE strain Hypr. In contrast to chick cells, the human cells showed the same activity at 4° and 37°C.

Discussion

The high antibody prevalence in host animals (10) from the areas studied caused us to expect TBE virus isolates from the thousands of *I. ricinus* collected in 1973–75. This was not the case, but UUK was isolated (11) though with much lower antibody rates. Partial explanations for this may be:

1. During these years tick collections were made during the late summer and autumn. It has been indicated that TBE activity may be highest in the early summer and UUK activity highest in the autumn (2, cited in 6).

UUK seems to be more temperature-resistant than TBE. Our handling of ticks collected in 1973–75 may have adversely affected the chances of TBE isolation (6). The ticks were kept in refrigerator for up to 14 days and transported to the laboratory at air temperature. The effect of such sudden temperature changes is not known to us.

In 1976 tick collections were made early in the season, and the ticks were either frozen immediately on dry ice or kept and transported at air temperature.

Isolate E 674 from Dragsvik in Sogn illustrates a potential public health hazard, since the ticks were collected on a camping ground, mostly from the vegetation between the cabins. Three out of five virus strains were from *male* ticks, thus demonstrating the ability of TBE viruses to pass interstadially in a vector.

The field expedition was supported by a grant from the *Nansen Fund*. Expert technical assistance was given by *Einar Brunvold and Hallgrim Søgnard*.

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Cell culture supernatants from BHK 21/c13 cultures were concentrated 300 times by polyethylene glycol 6000/NaCl (PEG/NaCl) precipitation, tested for infectivity in newborn mice, and also used as antigens in the serological identifications (13)

The haemagglutinating (HA) activities were tested and haemagglutination inhibition HAI) tests were performed with trypsinized human 0 (7) and newborn chick erythrocytes at *pH 6 0–6.2* and 4° and 37°C (3).

In addition to HAI serological identification and comparisons were performed by a standard complement fixation test (CFT) (4), gel precipitation in 1% agarose gel (closed hexagon immunodiffusion) (11) and immunoelectroosmophoresis (13)

TABLE 1 Ixodes ricinus *Pools Yielding TBE Virus Isolates, Collected June 8–11 1976*

Pool no	Collection site	Composition	Incubation time[a])				
			P in[b])	M1	M2	M3	Reisolation
E 672	Lavik in Sogn	9 ♀ ♀	3	3	3	3	a + b + [c])
E 674	Dragsvik in Sogn	12 ♂ ♂	4	3	3	3	a + b +
E 678	Bygstad, Nordfjord	6 ♂ ♂	5	3	3	3	a ND[d]), b +
E 681	Dragsvik	12 ♂ ♂	3	4	3	3	a ND b +
E 682	Dragsvik	4 ♀ ♀	5	3	3	3	a ND b +

[a]) Number of days from intracerebral inoculation until manifest signs of encephalitis in baby mice.
[b]) Primary inoculation and mouse brain passage *1 2 and 3*
[c]) a: December 1976 b: April 1978
[d]) Not done.

TABLE 2 *Serological Relationships of Norwegian Tick Isolates to TBE Virus, Strain Hypr[a])*

	HAI		CFT		CHI		IEOP	
Antigen	M	R	M	R	M	R	M	R
TBE Hypr SA	1280	320	512	128	Reactions of identity		8	4
TBE Hypr BHK	ND	ND	ND	ND	»		4	4
E 672 SA	640	320	512	128			4	2
E 672 BHK	ND	ND	ND	ND	»		4	2
E 674 SA	1280	320	256	128	»		8	4
E 674 BHK	ND	ND	ND	ND	»		4	4
E 678 SA	640	320	256	128	»		4	4
E 678 BHK	ND	ND	ND	ND	»		4	4
E 681 SA	640	320	256	64	»		4	2
E 681 BHK	ND	ND	ND	ND			4	2
E 682 SA	640	320	256	128			4	4
E 682 BHK	ND	ND	ND	ND	»		4	2

[a]) Negative control sera from mice and rabbits gave no reactions. Control antigens from uninfected mouse brains and cell cultures were negative in all tests

HAI Haemagglutination inhibition test
CFT Complement fixation test
CHI Closed hexagon immunodiffusion
IEOP Immunoelectroosmophoresis
M Mouse antiserum to TBE Hypr
R. Rabbit antiserum to TBE Hypr
SA antigen: Sucrose-acetone extracted suckling mouse brain
BHK: Concentrated BHK 21/c13 cell culture fluid
ND Not done

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BRIEF REPORT

CHLAMYDIA TRACHOMATIS FROM MEN WITH NON-GONOCOCCAL URETHRITIS SIMPLIFIED PROCEDURE FOR CULTIVATION AND ISOLATION IN REPLICATING McCOY CELL CULTURE

Peter A Csango

National Institute of Public Health, Department of Virology Oslo Norway

Csango, P A. *Chlamydia trachomatis* from men with non-gonococcal urethritis Simplified procedure for cultivation and isolation in replicating McCoy cell culture Acta path microbiol scand Sect. B, 86 257–259 1978

Chlamydia trachomatis was cultivated on replicating McCoy cells without the addition of antimetabolites A further technical modification was centrifugation of the specimens at room temperature at 4000 *g* thus making it possible to use the method in any microbiological laboratory *C trachomatis* was isolated from 36 of 81 patients (44 4%) with non-gonococcal urethritis This rate compares well with reported isolation rates using antireplicative agents and higher centrifugation temperatures

Key words *Chlamydia trachomatis*, urethritis

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Isolation of *Chlamydia trachomatis* in cell culture is a complicated procedure Chlamydial infections are common, and it would be a distinct advantage if the cell culture procedures were simplified Early diagnosis is important for differential diagnostic epidemiological and therapeutical reasons (5)

Not all diagnostic laboratories have the necessary equipment for techniques requiring an expensive temperature regulated high-speed centrifuge

All earlier methods have either made use of irradiated cells or an antimetabolite has been added to the cell culture medium (iododeoxyuridine, cytochalasin-B, cycloheximide) The action of these agents is complex and may not be reproducible (4) They are thought to control or stop cell growth thereby giving a selective advantage to *C trachomatis* The reagents may however induce latent viruses in cell cultures and constitute a potential biohazard to the laboratory staff It seemed desirable, therefore, to try out simple, rapid method avoiding the use of antimetabolites

Materials and Methods

Patient material From September 1977 a study was made of 81 men with non-gonococcal urethritis (NGU) attending the Department of Venereology of the Oslo Board of Health Patients were included if no Gram negative diplococci were present in a smear of urethral secretion and where *Neisseria gonorrhoeae* could not be cultivated from the secretion All the men were under 35 years of age and had not used antibiotics during the preceding 2 months

Collection and transport of specimens Intraurethral secretion was collected using an endourethral swab (E N T Swab Outfit, MW 142, Medical Wire and Equipment Co Potley Corsham, Wilts England) (2) Specimens were placed at 4° C in a plastic capsule containing an 0 23 M sucrose-phosphate medium (3 10) They reached the laboratory within 2–3 hours after collection

Cell culture Monolayers of conventional, untreated McCoy cells were prepared in Sterilin vials with screw caps (50 × 18 mm, Teddington, Middlesex, England)

containing a circular cover slip 13 mm in diameter (Chance propper Smethwick, Warley England). The vials were seeded with 2×10^5 cells (7) in 1 ml of medium 199 supplied with 10% calf serum and 0.5% glucose, vancomycin and streptomycin (5) (Vancomycin was a gift from Eli Lilly Norway). The cells were incubated overnight in a CO_2 incubator in a 5% CO_2 athosphere. The screw caps were loosened during incubation.

Isolation technique

Inoculation The technique was based on the system applied by *Darougar et al* (1) and *Johnson & Hobson* (5). Specimens were shaken on a Whirlimixer for 1 minute before being inoculated into vials. Aliquots of each specimen were added to two vials. The caps of the vials were tightened to prevent the escape of CO_2 and a change in pH. The inoculated vials were centrifuged at room temperature in a general laboratory centrifuge with an angle head rotor at 4000 *g* for 1 hour.

Incubation The cell cultures with the caps of the vials again loosened were incubated for 48–72 hours at 37° C.

Staining and microscopy After 48–72 hours incubation, the medium was removed from one vial, and the preparation was fixed with two changes of absolute methanol for 5 minutes. Methanol was replaced by freshly diluted Giemsa (9 parts tap water plus 1 part Giemsa), the preparation was stained for 30 minutes and then washed once in tap water. The preparation was washed in tap water for 1 minute, then dehydrated in acetone, drained and cleared in xylene, mounted in DPX and left for 15 minutes.

The preparations were screened by a Zeiss microscope by dark field microscopy.

A 10× objective lens was used, and the findings were confirmed with a 100× objective lens under oil immersion. The inclusion bodies showed a bright golden yellow colour while the cytoplasm and nuclei were pale brownish-green.

If the first culture was positive, the cells in the second culture were harvested and stored in a −70° C refrigerator for further studies. If the first vial was negative, the second vial was stained not later than 72 hours after incubation and examined for inclusion bodies.

Results and Discussion

During this study *C. trachomatis* was isolated for the first time in Norway. Of the 81 men with nongonococcal urethritis, 36 were infected with this organism (44.4%). The isolation rate is similar to that demonstrated in earlier studies (Table I). In the present study untreated McCoy cells have been used. Conflicting views exist as to whether non-replicating cells are really more effective than conventional monolayers for isolating *C. trachomatis*. It has been pointed out by several authors that *C. trachomatis* will grow also in untreated cells, provided that optimum conditions for each cell type are closely maintained (4, 5, 7). However, there are almost no clinical studies using replicating cells.

In this study replicating cells were used, thus avoiding antimetabolites and the dangers these drugs represent for the laboratory personnel. No overgrowth was experienced when the recommended cell suspension was used, and it was easy to see the inclusions by dark-ground microscopy.

The isolation procedure is also simplified by using room temperature, which seems to be adequate during centrifugation. Other techniques require a temperature of 30–35 C, which means using an expensive temperature regulated centrifuge.

Although *Johnson & Hobson* (5) found that 2500 *g* may be sufficient for use in clinical laboratories, they achieved the highest inclusion counts when using 4000 *g* during their study of a laboratory strain of *C. trachomatis*. The inclusion counts did not increase over this *g* value. 4000 *g* is attainable by a general laboratory centrifuge.

We feel that the clinical material presented here is a support for the use of replicating cells, and the procedure described should therefore facilitate the diagnosis of chlamydial oculogenital infections.

Thanks are due to Dr. C. H. Mordhorst, Statens Seruminstitut, Copenhagen, for valuable advice.

References. 1. *Darougar, S., Kinnison, J. R. & Jones, B. R.*: Simplified irradiated McCoy cell culture for isolation of Chlamydiae. In: *Nichols, I. C. S.* (Ed.) Trachoma and related disorders, No. 223. Excerpta Medica, Amster-

TABLE I *Isolation Rates (%) of* Chlamydia trachomatis *from Men with Non-gonococcal Urethritis, Centrifugation Temperature (T), Relative Centrifugal Force* (g) *(RCF) and Treatment of McCoy Cell Cultures Before or During Incubation*

Treatment of cells	Isolation rates (%)	T	RCF	Reference
x-ray irradiation	39	35	2500	(8)
x-ray irradiation	44.5	35	2700	(3)
x-ray irradiation	42	30–35	3000	(9)
iododeoxyuridine	49	35	2750	(6)
untreated	44.4	20	4000	(present study)

don and New York 1970 p. 63 – 2. *Dunlop E M C Vaughan-Jackson, J D & Darougar S* Br J vener Dis 48 521–424 1972a – 3 *Dunlop E M C Vaughan Jackson, J D Darougar S., & Jones, B R* Br J, vener Dis 48 425–428, 1972b. – 4 *Hobson, D* Tissue culture procedures for the isolation of *Chlamydia trachomatis* from patients with nongonococcal genital infections In *Hobson, D and Holmes, K K* (Eds) Nongonococcal urethritis and related Infections American Society for Microbiology Washington 1977 pp 286–294 – 5 *Johnson, F W A & Hobson, D* J Hyg (Camb) 76 441–451 1976 – 6 *Oriel, J D Reeve P Wright, J T & Owen, J* Br J vener Dis 52 46–51 1976 – 7 *Richmond, S* Lancet *1* 965 1976 – 8. *Richmond, S J Hilton, A. L & Clarke S K R.* Br J vener Dis. 48 434–444 1972 – 9 *Schachter J Hanna, L Hill, C E Massad, S Sheppard, C W Conte, J E Cohen, S N & Meyer K F* J A. M A. 231 1252–1255 1975 – 10 Guide to the laboratory diagnosis of trachoma World Health Organization, Geneva 1975 1–38

containing a circular cover slip 13 mm in diameter (Chance propper Smethwick, Warley England). The vials were seeded with 2×10^5 cells (7) in 1 ml of medium 199 supplied with 10% calf serum and 0 5% glucose, vancomycin and streptomycin (5). (Vancomycin was a gift from Eli Lilly Norway) The cells were incubated overnight in a CO_2 incubator in a 5% CO_2 athosphere. The screw caps were loosened during incubation.

Isolation technique

Inoculation The technique was based on the system applied by *Darougar et al.* (1) and *Johnson & Hobson* (5). Specimens were shaken on a Whirlimixer for 1 minute before being inoculated into cials. Aliquots of each specimen were added to two vials. The caps of the vials were tightened to prevent the escape of CO_2 and a change in pH The inoculated vials were centrifuged at room temperature in a general laboratory centrifuge with an angle head rotor at 4000 *g* for 1 hour

Incubation The cell cultures with the caps of the vials again loosened were incubated for 48–72 hours at 37° C.

Staining and microscopy After 48–72 hours incubation, the medium was removed from one vial, and the preparation was fixed with two changes of absolute methanol for 5 minutes. Methanol was replaced by freshly diluted Giemsa (9 parts tap water plus 1 part Giemsa) the preparation was stained for 30 minutes and then washed once in tap water The preparation was washed in tap water for 1 minute, then dehydrated in acetone, drained and cleared in xylene, mounted in DPX and left for 15 minutes.

The preparations were screened by a Zeiss microscope by dark-field microscopy

A 10 × objective lens was used and the findings were confirmed with a 100 × objective lens under oil immersion. The inclusion bodies showed a bright golden-yellow colour while the cytoplasm and nuclei were pale brownish-green

If the first culture was positive, the cells in the second culture were harvested and stored in a −70° C refrigerator for further studies If the first vial was negative the second vial was stained not later than 72 hours after incubation and examined for inclusion bodies

Results and Discussion

During this study *C trachomatis* was isolated for the first time in Norway Of the 81 men with nongococcal urethritis, 36 were infected with this organism (44 4%) The isolation rate is similar to that demonstrated in earlier studies (Table 1). In the present study untreated McCoy cells have been used Conflicting views exist as to whether non-replicating cells are really more effective than conventional monolayers for isolating *C trachomatis* It has been pointed out by several authors that *C trachomatis* will grow also in untreated cells, provided that optimum conditions for each cell type are closely maintained (4 5 7) However there are almost no clinical studies using replicating cells

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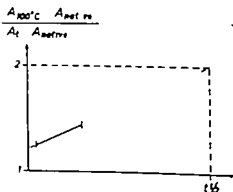
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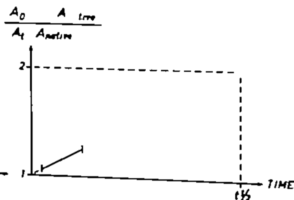
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Vol. 86 B, Fasc. 2. April 1978

On page 72 fig. 1 beneath the headline
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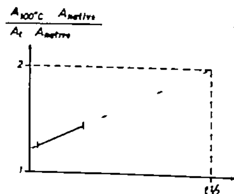
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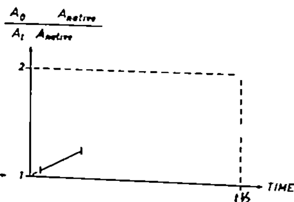
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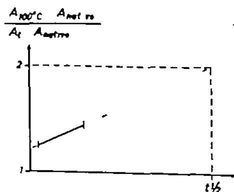
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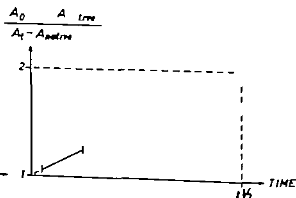
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Acta path. microbiol. scand. Sect. B, 86 261–266, 1978.

PREVALENCE OF NON-CHOLERA VIBRIOS IN CAVUM NASI AND PHARYNX OF DUCKS

M. BISGAARD, R. SAKAZAKI and T. SHIMADA

Institute of Avian Diseases, Dept. Langå, Ryevej 1, DK-8870 Langå, Denmark and The first Department of Bacteriology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Bisgaard, M., Sakazaki, R. & Shimada, T. Prevalence of non-cholera vibrios in cavum nasi and pharynx of ducks. Acta path. microbiol. scand. Sect. B, 86 261–266, 1978.

Investigations among ducks on two different farms showed a high prevalence of *Vibrio cholerae* in cavum nasi and pharynx after the ducks were admitted to the open field. In no case was *Vibrio cholerae* isolated from ducks which had never been outside the houses. At least six serovars were isolated, O 54 being the most prevalent. All the strains isolated produced a destructive cytotonic effect in Y1 adrenal cells and caused fluid accumulation in rabbit gut loops. Migratory birds were incriminated as the source of initial contamination.

Key words: Vibrio cholerae, non-cholera vibrios, gastroenteritis, ducks.

Magne Bisgaard, Institut for fjerkræsygdomme, afdeling Langå, Ryevej 1, DK-8870 Langå.

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In recent years vibrios similar to cholera vibrios have attracted increasing attention. They cannot be distinguished from each other except by their somatic antigens. *Sakazaki et al.* (1967, 1970) classified them into *Vibrio cholerae*, in which serovar 1 was assigned to cholera vibrio and other serovars to so-called non-cholera vibrio (NCV) or non-agglutinable vibrio (NAG). Non-cholera vibrios, however, are capable of causing disease resembling cholera (*Gaines et al.* 1964, *McIntyre et al.* 1965, *Chatterjee & Neogy* 1971, *Ko et al.* 1973, *Bäck et al.* 1974, *Yacesen et al.* 1974, *Pokrovsky et al.* 1974) and have also been connected with a type of food poisoning (*Aldová et al.* 1968, *Dakin et al.* 1974, *Zakhariev et al.* 1976).

Enterotoxic activity resembling that of cholera vibrio has been demonstrated in cell-free culture supernatant fluid from NCV (*Ohashi et al.* 1972, *Zinnaka & Carpenter* 1972).

The role of animals in the maintenance of infections caused by cholera vibrio is not known. The observation that animals were infected for longer periods than humans, and for longer than the environment was contaminated, points to a possible role played by animals (*Sanyal et al.* 1974). Also hydrobionts may take part in the maintenance of the endemicity (*Avtsyn et al.* 1976).

The isolation of NCV from ducks and their surroundings in Denmark (*Bisgaard & Kristensen* 1975) raised questions as to the source of infection, the prevalence of NCV among clinically normal ducks of different ages, and to what extent strains isolated are toxinogenic. The report deals with these questions.

MATERIAL AND METHODS

Two duck farms known to be infected with *V. cholerae* (non-cholera vibrios) were selected. Day-old ducks were placed in heated houses where they spent the first two weeks of their lives after which they were moved to an open field without natural pools or streams. The ducks had free access to a commercial feed-mixture and to water from a drinking source. From June to September ducks were received weekly from three different flocks on these two farms, and swabs from cavum nasi and pharynx were examined for the presence of *V. cholerae*

One side of the swab was smeared over one-fifth of a blood agar plate, the rest of the plate being inoculated with a loop carrying material from the smeared portion. After overnight incubation at 37°C, the qualitative composition of the bacterial flora was determined on the basis of morphology and primary diagnostic tests (*Cowan & Steele* 1970 *Hugh & Sakazaki* 1972). Colonies typical of *V cholerae* were subcultured and examined as described previously (*Bisgaard & Kristensen* 1975) *V cholerae* was expressed as percentage of the total number of colonies. Ducks routinely received for post mortem examinations during the same period from the farms mentioned and from other farms were subjected to the same tests. 110 NCV were isolated and characterized biochemically

Investigations regarding serovar determination were performed according to *Sakazaki et al* (1970) and *Shimada & Sakazaki* (1977) and 105 strains were examined.

All but five cultures were tested for LT enterotoxin by Y1 adrenal cell tissue culture assay (*Sack & Sack* 1975) and for ST enterotoxin by infant mice assay (*Dean et al.* 1972) The same cultures were tested for vascular permeability factor in rabbit skin (*Craig* 1966) and for ability to cause fluid accumulation in rabbit gut loops (*De & Chatterjee* 1953)

TABLE 1 *Prevalence of* V cholerae *Serovars in Cavum Nasi and Pharynx of Ducks of Different Ages*

No of ducks examined	Flock no.	Age in weeks	No. of ducks with *V cholerae*/ No examined: cavum nasi	pharynx	*V cholerae* serovars isolated	No. of ducks in which *V Cholerae* form: <50%	≥50% <90%	≥90% of the colonies
5		day-old	0/5	0/5	–			
5	1	1	0/5	0/5	–			
5	2	1	0/5	0/5	–			
5	3	1	0/5	0/5	–			
5	1	2	3/5	3/5	0-34	Not determined		
5	2	2	0/5	0/5	–			
5	3	2	1/5	1/5	0-60	1^{a} 1^{p}		
5	1	3	5/5	5/5	0-34	2 5^{p}	2^{a}	1
5	2	3	5/5	5/5	0 34	1 4^{p}	1^{p}	4
5	3	3	2/2	3/3	0 60	3^{p}	1^{a}	1
4	1	4	4/4	4/4	0 54	1 2^{p}	2^{p}	3^{a}
4	2	4	4/4	4/4	0 34 R, Unknown	4^{p}	4^{a}	
5	3	4	5/5	5/5	0 2, 0 60	2 5^{p}	1^{a}	[illegible]
4	1	5	4/4	4/4	0 54	3^{p}	4 1^{p}	
4	2	5	4/4	4/4	0-2, 0 54	4^{p}	2	2
5	3	5	3/4	1/4	0 34 0 60	2 1^{p}		1^{a}
4	1	6	4/4	4/4	0 34 R, Unknown	4^{p}	4	
3	2	6	3/3	3/3	0 2 0 54	3 3^{p}		
4	1	7	4/4	2/2	0 54 0 57 Unknown	4 2^{p}		
4	2	7	3/3	3/3	0 2, R, Unknown	Not determined		

No of asterisks indicate the no of blood agar plates discarded owing to *Proteus* swarming

^{a} Cavum nasi

^{p} Pharynx

R Rough antigen form

RESULTS

Considerable changes in the bacterial flora in cavum nasi and pharynx were connected with the admittance of the ducks to the open field. Mainly quantitative differences were noted between the bacterial flora of cavum nasi and pharynx within the same age-groups.

The prevalence of *V. cholerae* in cavum nasi and pharynx of ducks of different ages is shown in Table 1. In no case was *V. cholerae* isolated from ducks which had never been outside the houses. After the ducks were admitted to the open field, the prevalence of *V. cholerae* was very high and colonies typical of *V. cholerae* often dominated the bacterial flora, especially as far as cavum nasi is concerned (Table 1). Bacterial species belonging to the following genera could be isolated with varying incidence from cavum nasi and pharynx: *Actinomyces, Bacillus, Corynebacterium, Lactobacillus, Micrococcus, Staphylococcus, Streptococcus, Acinetobacter, Achromobacter, Alcaligenes,* Enterobacteriaceae*, Flavobacterium, Moraxella, Neisseria, Pasteurella, Pseudomonas* and *Vibrio*. Investigations involving ducks from other farms located far away from each other showed that *V. cholerae* could be isolated from ducks received from these farms also (Table 2).

Biochemical reactions of the strains of *V. cholerae* isolated are shown in Table 3. Nine out of 110 strains examined belonged to Heiberg's biovar I, the rest to biovar II. 93% of the glycerol-positive strains and 89% of the lactose-positive strains showed delayed reactions.

Not less than six different serovars were isolated (Table 4), 0.54, 0.34 and 0.60 being the most prevalent. As far as flock nos. 1 and 2 are concerned a change from serovar 0.34 to 0.54 and 0.54 to 0.2 was noted at the age of 4 and 5 weeks, respectively (Table 1). On the other hand 0.60 was isolated constantly from flock no. 3. At least two other serovars were present (Table 1). As can be seen from Table 2, 0.54 was isolated from nine out of ten farms investigated, while 0.34 and 0.60 were present on five and four farms, respectively. In five out of 29 ducks from which *V. cholerae* was isolated from both cavum nasi and pharynx, different serovars were detected.

All the strains isolated induced a distinctive cytotoxic effect on Y1 adrenal cells. Affected cells were round, and many of them floated freely in the medium. The cytotoxic effect of the culture filtrate was destroyed by heating at 100°C for 15 minutes but was not neutralized with cholera antitoxin. However, culture filtrates induced fluid accumulation in rabbit ileal loops. Some haemorrhagic effect was demonstrated in the rabbit skin tests.

ST enterotoxin was not demonstrated in any of the culture filtrates.

TABLE 2. *Prevalence of* V. cholerae *Serovars in Ducks Received from Different Farms*

Farm	No. of submittings	No. of ducks examined	No. of culture-positive ducks	*V. cholerae* serovars isolated
A	15	63	44	0.2, 0.34, 0.54, 0.57, 0.60, unknown, R
B	7	33	16	0.2, 0.34, 0.54, 0.60
C	1	2	1	0.54
D	1	4	4	0.34, 0.54
E	1	4	3	0.34, 0.54
F	1	4		0.54
G	2	6	2	0.54
H	1	2	2	0.34, 0.60
I	2	10*	6	0.54, 0.60
J	1	4	1	0.54

No. of asterisks indicates the no. of ducks examined bacteriologically and in which swarming of *Proteus* prevented isolation of *V. cholerae*.

R. Rough antigen form.

TABLE 3 *Biochemical Characters of 110 Strains of* V *cholerae*

	Sign[a]	% positive		Sign	% positive
Motility	+	100	Glycerol, acid	(d)	83
Catalase	+	100	Adonitol acid	–	0
Oxidase	+	100	Arabinose, acid	–	0
Oxidation/Fermentation	F	100	Xylose, acid	–	0
H_2S/TSI	–	0	Dulcitol, acid	–	0
Haemolysis	+	100	Inositol acid	–	0
KCN	–	0	Mannitol acid	+	100
Malonate	–	0	Sorbitol acid	–	0
VP 37°C	d	49	Fructose acid	+	100
Nitrate	+	100	Galactose, acid	+	100
Urease	–	0	Glucose, acid	+	100
Arginine	–	0	Glucose, gas	–	0
Lysine	+	100	Mannose, acid	–	8
Ornithine	+	100	Rhamnose, acid	–	0
Phenylalanine	–	0	Cellobiose, acid	–	2
Indol	+	97	Lactose, acid	(+)	96
Gelatinase	+	100	Maltose, acid	+	100
Starch, hydrolysis	+	100	Melibiose, acid	–	0
Citrate Simmons	+	100	Sucrose, acid	+	100
Mucate	–	0	Trehalose, acid	+	100
Pellicle formation	d	87	Raffinose, acid	–	0
Pigment	–	6	Dextrin, acid	+	100
Pteridine sensitivity	+	100	Inulin acid	–	0
3% NaCl, growth	+	100	Salicin acid	–	0

[a] + = most (90% or more) strains positive
– = most (90% or more) strains negative
d = some (less than 90%) strains positive, some negative
F = fermentation
() = figures in brackets indicate delayed reactions (3 or more days)

TABLE 4 *Distribution of 105 Strains of* V *cholerae According to Serovar*

Serovar	0 2	0 34	0·54	0 57	0 60	Unknown	R form
No of strains	7	27	37	1	20	8	5

R Rough

DISCUSSION

Morphological and biochemical characteristics of the strains of *V cholerae* isolated are in accordance with those reported by *Sakazaki et al* (1967) except as regards mucate. On the other hand *Aldova et al* (1968) and *Nacescu et al* (1974) describe only mucate-negative strains of NCV Formation of a water soluble brown pigment has never been described earlier as far as is known to the authors *Chatterjee* (1974) found that NCV belonged to Heiberg s biovars I II and V Only biovars I and II were present in our strains. The presence of a single serovar (0 54) on nine out of ten different farms at long distances from each other indicates a very high prevalence of this serovar not least because only one submitting was received from six of the ten farms An increased number of serovars was detected the more submittings there were from a farm On the other hand subculturing of more than one colony typical of *V cholerae* from cavum nasi and pharynx might have disclosed ducks with

different serovars in both cavum nasi and pharynx.

The source of initial contamination of the surroundings is not known. However contamination is unlikely to have originated from human sources through contaminated waste water. Since it was not possible to isolate NCV from ducks kept in houses provided with water from the same drinking water source as in the field, migratory birds foraging in NCV contaminated waste and surface water could be incriminated as the source of infection, as suggested earlier by *Bisgaard & Kristensen* (1975). Investigations to confirm this will be carried out.

Thus NCV in our area seems to be maintained by a cycle of transmission from bird to bird through the environment. These observations, together with the findings of *Artyn et al.* (1976), that infected tadpoles excreted NCV over a long period, strongly suggest that ecological interrelations between NCV and their surroundings are important factors in the epidemiology of these infections.

Dunaev et al. (1975) showed that NCV isolated from water and healthy persons possessed no enteropathogenic properties. However a positive rabbit gut loop test was obtained by *Draskovicová et al.* (1977) in 12 out of 16 NCV isolated from cases of diarrhoea and 12 out of 20 NCV isolated from water. All our strains isolated from cavum nasi and pharynx of ducks were toxinogenic in rabbit ileal loop tests. Although NCV seem to be relatively poorer producers of enterotoxin than the 0 1 serovar it should be considered that the factors responsible for enterotoxin production *in vitro* and *in vivo* are not yet known (*Draskovicová et al.* 1977). A positive rabbit skin test was reported by *Ohashi et al.* (1972) with non-agglutinable strains of *V. cholerae* isolated from human cases of diarrhoea. Some haemorrhagic effect was shown also by our strains. Unheated, cross-contaminated foods, combined with bad environmental sanitation and storage, may constitute a potential risk of food infection caused by NCV. Taking these things into consideration, NCV may well play an important part in the etiology of unknown cases of human diarrhoeal disease, since these organisms may be discarded by the usual enteric diagnostic procedures directed mainly towards isolation and identification of enteropathogenic members of *Enterobacteriaceae*.

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STATISTICAL ASPECTS OF THE TREPONEMAL COUNTS IN THE TPI TEST

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A method of counting treponemes in randomly selected visual fields is described. It was found that *Treponema pallidum* is distributed as a Poisson distribution among the visual fields and also among samples drawn from the same suspension. Immobilized treponemes are binomially distributed among the total number of treponemes. Application of the parameters of the Poisson and the binomial distributions should be made in order to determine the dosage of *T. pallidum* in animal experimentation and to evaluate the outcome of the TPI test. Some statistical aspects of the investigation of treponemal survival are presented.

Key words: TPI test, poisson distribution, binomial

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The *Treponema pallidum* Immobilization test (TPI) was the first serological test employed in treponemal diseases, using the pathogenic treponeme as antigen. The originators of the test, *R. A. Nelson Jr. & Manfred Mayer* (6), assumed that the treponemes were immobilized by a single antibody reacting with complement. For that reason, the TPI test is usually considered to be more specific than any other serological test for treponemal diseases. This opinion is shared by most authors. The outcome of the TPI test is expressed as the proportion of immobilized treponemes found. Two tubes are used for each serum, one containing unheated complement in addition to the treponemes and the serum, and one containing heated instead of unheated guinea pig serum. The latter tube serves as control. The counting of treponemes is therefore an essential part of the test procedure. The precision with which the proportion of immobile treponemes can be determined depends on the numbers of both motile and immobile organisms counted. How many it will be necessary to count depends on the manner in which the treponemes are distributed in the suspension on the slide. It could be expected that the treponemes were distributed as a Poisson distribution in analogy with what has been found with other organisms which neither attract nor repel each other, such as cells in a counting chamber, *»Student«* (11).

Analogous with games of chance, it could also be expected that the distribution of the immobilized treponemes among the total number of treponemes would be very much like a binomial distribution. The investigation by *Nielsen* (8) of the reproducibility of the test was based on this assumption.

MATERIAL AND METHODS

The treponemes were eluted from syphilitic rabbit orchitis and the elutions cleared of debris etc. They were then mixed with serum and evacuated as described by *Nielsen* (8, 9), except for the following: After the clearing centrifugation (900 g), the suspension was transferred to a clean 40 ml roundbottom centrifuge tube and shaken in a machine at 150 strokes/minute, 2 cm stroke length for

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STATISTICAL ASPECTS OF THE TREPONEMAL COUNTS IN THE TPI TEST

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A method of counting treponemes in randomly selected visual fields is described. It was found that *Treponema pallidum* is distributed as a Poisson distribution among the visual fields and also among samples drawn from the same suspension. Immobilized treponemes are binomially distributed among the total number of treponemes. Application of the parameters of the Poisson and the binomial distributions should be made in order to determine the dosage of *T. pallidum* in animal experimentation and to evaluate the outcome of the TPI test. Some statistical aspects of the investigation of treponemal survival are presented.

Key words: TPI test; poisson distribution, binomial.

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The *Treponema pallidum* immobilization test (TPI) was the first serological test employed in treponemal diseases, using the pathogenic treponeme as antigen. The originators of the test, *R. A. Nelson Jr. & Manfred Mayer* (6), assumed that the treponemes were immobilized by a single antibody reacting with complement. For that reason, the TPI test is usually considered to be more specific than any other serological test for treponemal diseases. This opinion is shared by most authors. The outcome of the TPI test is expressed as the proportion of immobilized treponemes found. Two tubes are used for each serum, one containing unheated complement in addition to the treponemes and the serum, and one containing heated instead of unheated guinea pig serum. The latter tube serves as control. The counting of treponemes is therefore an essential part of the test procedure. The precision with which the proportion of immobile treponemes can be determined depends on the numbers of both motile and immobile organisms counted. How many it will be necessary to count depends on the manner in which the treponemes are distributed in the suspension on the slide. It could be expected that the treponemes were distributed as a Poisson distribution in analogy with what has been found with other organisms which neither attract nor repel each other, such as cells in a counting chamber, »Student« (11).

Analogous with games of chance, it could also be expected that the distribution of the immobilized treponemes among the total number of treponemes would be very much like a binomial distribution. The investigation by *Nielsen* (8) of the reproducibility of the test was based on this assumption.

MATERIAL AND METHODS

The treponemes were eluted from syphilitic rabbit orchitis and the elutions cleared of debris etc. They were then mixed with serum and evacuated as described by *Nielsen* (8, 9), except for the following: After the clearing centrifugation (900 g), the suspension was transferred to a clean 40 ml roundbottom centrifuge tube and shaken in a machine at 150 strokes/minute, 2 cm stroke length for

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TABLE 1 *Distribution of* Treponema pallidum *between the Visual Fields Agrees with the Assumption that They are Poisson Distributed*

Date	Vol. (μl) counted	Visual fields	Treponemes counted	$\bar{x}$	s^2	$s^2/\bar{x}$	χ^2	f	Probability of χ^2 (%)
7/1-76	0.1172	50	132	2.64	3.26	1.2348	60.5	49	80–90
	0.1149	49	119	2.34	2.75	1.1752	54.3	48	70–80
	0.1172	50	71	1.42	1.27	0.8944	43.8	49	30–40
21/4-76	0.0586	25	65	2.60	2.25	0.8654	20.7	24	40
	0.0586	25	57	2.28	2.29	1.0044	24.1	24	50–60
	0.0586	25	75	3.00	2.25	0.7500	18.0	24	20–30
	0.0586	25	86	3.44	2.26	0.6579	15.7	24	10–20
	0.0586	25	85	3.40	2.17	0.6382	15.3	24	10–20
	0.0586	25	66	2.64	3.41	1.2916	31.0	24	80–90
	0.0586	25	66	2.64	2.41	0.9234	22.2	24	40–50
	0.0586	25	110	4.40	2.67	0.6068	14.6	24	5–10
27/9-77	0.0586	25	68	2.72	3.13	1.1495	27.6	24	70–80
	0.0586	25	106	4.24	4.69	1.1061	26.5	24	60–70
	0.0586	25	114	4.56	4.67	1.025	24.5	24	50–60
	0.0586	25	167	6.68	6.14	0.9196	22.07	24	40–50
	0.0586	25	179	7.16	7.39	1.0321	24.8	24	50–60
	0.0586	25	81	3.24	4.69	1.4475	34.7	24	90–95
	0.0586	25	64	2.56	2.42	0.9466	22.7	24	40–50
	0.0586	25	110	4.40	6.17	1.4015	33.6	24	90–95
	0.0586	25	126	5.04	3.62	0.7189	17.25	24	10–20
	0.0586	25	94	3.76	4.52	1.2030	28.8	24	70–80
	0.0586	25	123	4.92	5.74	1.1673	28.0	24	70–80
	0.0586	25	138	5.52	5.51	0.0081	23.9	24	50–60
29/9-77	0.0586	25	68	2.72	2.29	0.832	19.9	24	30–40
	0.0586	25	96	3.84	4.06	1.0575	25	24	60–70

with the assumption that they were Poisson distributed (Chi-square 2.35, degrees of freedom 4, probability 30–40 per cent). Analogous counts have been performed on the crude suspension obtained immediately after the treponemes had been eluated from the orchitis, in order to investigate whether or not agglutination had taken place. The treponemes appeared to be Poisson distributed.

Distribution of the Immobilized Treponemes

Knowledge of the mathematical model which adequately describes the distribution of the immobilized treponemes makes it possible to determine the precision with which the proportion of immobiles has been determined. It also furnishes information as to whether agglutination has taken place during the immobilization experiment.

A number of treponemal counts were made on samples drawn from mixtures of treponema suspensions and syphilitic serum with active complement added. The mixtures had been incubated anaerobically at 35 C for 18 hours prior to the counting and the tubes were shaken before each count, as described under methods. The outcome of the 24 counts is shown in Table 5. Counting was performed in randomized visual fields as described. The number of immobile treponemes was counted in a predetermined fixed number. As it is necessary to count all treponemes in the visual fields examined, this number can only be approximate. The mean number of immobile treponemes per collection was determined and the binomial variance computed, bin. var = M(1-M/T). Here M is the mean number and T the number in the collection. Table 5 shows the date of each of the 24 counts, the number of groups of treponemes in each, the mean

10 minutes, before being distributed into the tubes with the liquids to be tested.

Reagents and nutrients. Guinea pig serum was used as complement. The animals were bled by heart puncture. Both freshly prepared guinea pig serum and serum which had been frozen at −20° C for less than 3 weeks were employed. The sera tested were syphilitic rabbit serum, non-reactive serum from non-syphilitic rabbits, and some human sera from the daily routine of the TPI laboratory

Instruments Carl Zeiss binocular microscope with graduated movable stage was used for counting at a magnification of 500× An oil immersion plane apochromatic objective 40× 1 00 mm num. aperture and oculars 12× were employed. The diameter of the visual field was 0 44 mm measured with an object micrometer and the area was calculated to be 0 152 mm^2 Using a Carlsberg pipette, samples of 5 µl were deposited on the slides and covered with a *18 × 18 mm*, coverslip Air bubbles were not allowed to be present in the preparations.

Counting method The number of treponemes in 25 or 50 visual fields was counted and the number of immobilized treponemes recorded. Each visual field was examined in its full depth using the fine adjustment head. The visual fields were chosen according to a random procedure In order to loclocate each visual field, two numbers were selected at random using a table of random numbers. Each number was one of two rectangular co-ordinates. The origin of the co-ordinate system was obtained as the two numbers of the scales of the mechanical stage which corresponded to the location of the farther left hand corner of the coverslip when the objective was focused there. Focusing of the visual fields was obtained by adding or subtracting the numbers selected for the visual field from the co-ordinates of the origin. An example of distribution is shown in Fig. 1 The table of random numbers used was Table XIX by *Hald* (2). The writer performed all the counts and carried out all calculations with a Hewlett Packard pocket calculator No. 21

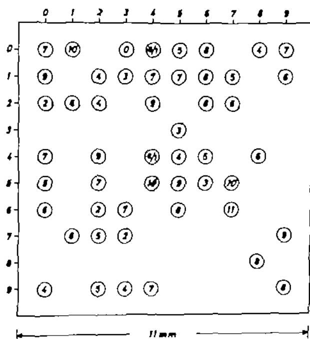


Fig 1 The figures along the upper horizontal line and along the left hand perpendicular line indicate the numbers on the scales of the mechanical stage The circles filled with numbers are the visual fields examined The figures within the circles are the number of treponemes counted in each visual field If two numbers separated by an oblique bar are found, the number to the left of the line = number of treponemes counted and the number to the right = number of immobile treponemes.

RESULTS

Distribution of the Treponemes between the Visual Fields

A series of 25 counts made at varying intervals over a period of 1 ½ years is shown in Table 1 The distribution of the treponemes among the visual fields in the slide preparations is shown. The mean number ($\bar{x}$) of treponemes per visual field was determined for each serum and the variance (s^2) of the distribution was determined from the figures. The mean value of treponemes per visual field is an estimate of the hypothetical Poisson parameter (λ) this parameter is at the same time the mean and the variance of the Poisson distribution. The ratio of the variance actually found and the estimate of the Poisson variance were computed.

The ratio $s^2/\bar{x}$ multiplied by the number of degrees of freedom f given in column 9 should be distributed approximately as a Chi-square. Column 10 shows that good agreement was found between the figures observed and a Chi-square distribution.

A number of counts have been made in order to check the distribution of the treponemes if the tube was left standing for some time in the laboratory the distribution of the treponemes in repeated samples from the same tube, and the influence of shaking the tubes.

Table 2 shows the distribution of the treponemes among various samples taken from the same tube. The tubes were shaken before each count. It can be seen that the treponemes are distributed in a manner which conforms to the Poisson distribution

Table 3 shows counts of treponemes in samples taken from the same tube from the bottom at varying times and leaving the tube undisturbed The tube was left for 140 minutes. The treponemes were Poisson distributed among the samples. A slight increase in immobile treponemes can be seen.

In Table 4 the effect of shaking the suspension in various ways was examined by comparing with a count made before shaking. The variation in number of treponemes between samples agreed well

TABLE 5 *Distribution of Immobilised* Treponema pallidum *among the Motile Treponemes*

Date	n	S(x)	Immob trep	M	Bin. var	s^2	s^2/bin. var	χ^2	f	Probability of χ^2 (%)
4/11 75	4	100	25	6.25	4.69	14.25	3.038	9.12	3	95–97.5
5/11	4	100	30	7.50	5.25	7.00	1.333	3.99	3	70–80
5/11	4	100	17	4.25	3.53	11.58	3.28	9.80	3	97.5
6/11	4	100	22	5.50	4.29	3.00	0.699	2.09	3	20–30
6/11	4	100	27	6.75	4.92	4.92	1.000	3.00	3	60–70
7/11	4	100	18	4.50	3.69	4.33	1.734	3.52	3	60–70
7/11	4	100	25	6.25	4.69	2.92	0.6226	1.87	3	30–40
1/4 76	6	180	5	0.83	0.805	1.71	2.124	10.62	5	90–95
27/1	8	192	29	3.63	3.086	3.98	1.289	9.03	7	70–80
11/6	8	239	133	16.63	7.41	6.84	0.923	6.46	7	50–60
10/6	4	64	48	12.00	3.00	2.66	0.8867	6.21	3	80–90
30/6	8	214	35	4.38	3.679	2.55	0.6931	4.85	7	30–40
15/7	4	83	69	17.25	3.105	1.58	0.5088	1.52	3	30–40
15/	6	120	27	4.50	3.534	6.70	1.8959	9.48	5	90–95
28/7	8	214	35	4.38	3.68	14.23	3.866	9.48	7	90–95
10/9 77	4	198	116	29.0	11.89	2.00	0.1682	0.504	3	5–10
10/9	2	81	48	24.0	9.84	18.00	1.8292	1.83	1	80–90
10/9	2	100	73	36.5	9.96	24.50	2.48	2.48	1	80–90
10/9	2	192	29	14.50	12.33	0.500	0.0406	0.0406	1	10–20
10/9	3	125	75	25.00	9.75	9.080	0.9231	1.846	2	60–70
10/9	3	114	65	21.67	9.32	24.340	2.6111	5.22	2	90–95
20/10	9	638	174	19.33	14.11	11.25	0.7973	6.38	8	40–50
20/10	24	256	68	6.18	2.719	2.23	0.8201	18.86	23	20–30
20/10	25	250	70	2.80	2.016	2.916	1.4464	34.7	24	90–95

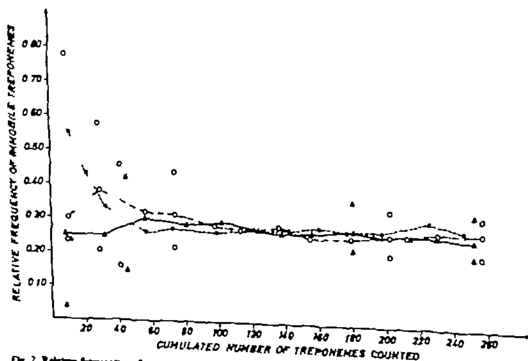


Fig 2 Relative frequencies of immobile treponemes found in continuous counts of organisms in three samples from the same tube The points outside the drawn graphs represent 95 per cent confidence limits corresponding to the counts

TABLE 2. *Distribution of* Treponema pallidum *in Repeated Samples from same Tube The Tube was Shaken before each Count*

Date	No. of samples	Vol. counted in each (µl)	Visual fields counted	Total number treponemes	Treponemes per sample = X	s^2	s^2/X	χ^2	f	Probability of χ^2 (%)
11/11 76	4	0 0586	25	180	45	32.67	0 726	2.18	3	40–50
27/9	5	0 0586	25	1191	238 2	494 4	2 07	8.28	4	90–95
9/8 77	6	0 0586	25	1465	244 17	158.57	0 6494	3 25	5	30–40
13/9 77	8	0 0586	25	545	68 13	114 95	1 6876	11 8	7	80–90
15/9 77	4	0 1172	50	1097	274 25	355 6	1 2966	3 89	3	70–80
27/9	4	0 0586	25	166	41.50	71 0	1 7108	5 13	3	80–90

Taking X as an estimate of the Poisson parameter it can be seen that the treponemes are distributed among the samples as a Poisson distribution.

TABLE 3 *Distribution of Samples Taken from the same Tube Left Undisturbed in the Atmosphere of the Laboratory*

Time (min)	Number of visual fields examined	Number of treponemes	Number of immobile treponemes
0	25	264	4
20	25	245	2
50	25	240	4
65	25	226	0
72	25	240	4
140	25	250	16

number M of immobiles per sample (column 5), the variance computed from the figures in each sample: s^2 (column 7), and the number of degrees of freedom f (column 10). A method analogous with that described for Table 1 for the assessment of agreement between the hypothetical distribution and that observed was used. With the exception of two observations good agreement was found. The two divergent observations are from the 10th September 1977 They are unexplained. The findings recorded in Table 5 support the assumption that the probability of finding ~~was~~ immobile treponemes in a

TABLE 4 *Comparison between Samples Taken from the same Tube after various Ways of Shaking*

Type of agitation	Volume counted (µl)	Number of treponemes in volume	Number of immobile treponemes	Relative frequency of immobile treponemes
None	0 0586	40	15	0 39
By suction w Pasteur pipette	0 0586	49	23	0.42
By hand	0.0586	44	20	0 45
By machine	0 0586	54	24	0 44
By machine	0 0586	44	20	0.45
		231	102	0.44

The variation in number of treponemes between samples agreed well with the assumption that it was a Poisson distribution (χ^2 2 35 probability 30–40 per cent, f = 4). The immobilized treponemes were binomially distributed

TABLE 5 *Distribution of Immobilized* Treponema pallidum *among the Motile Treponemes*

Date	n	Σ(n)	Immob. trep.	M	Bin var	s^2	s^2/bin var	χ^2	f	Probability of χ^2 (%)
4/11 75	4	100	25	6.25	4 69	14 25	3 038	9 12	3	95–97.5
5/11	4	100	30	7.50	5.25	7.00	1.333	3 99	3	70–80
5/11	4	100	17	4 25	3.33	11.58	3.28	9 80	3	97.5
6/11	4	100	22	5 50	4 29	3 00	0 699	2.09	3	20–30
6/11	4	100	27	6 75	4 92	4 92	1 000	3.00	3	60–70
7/11	4	100	18	4.50	3 69	4.33	1 734	3.52	3	60–70
7/11	4	100	25	6 25	4 69	2 92	0 6226	1 87	3	30–40
1/4 76	6	180	5	0 83	0 805	1 71	2.124	10.62	5	90–95
27/1	8	192	29	3 63	3.086	3.98	1.289	9 03	7	70–80
11/6	8	239	133	16 63	7 41	6 84	0.923	6 46	7	50–60
10/6	4	64	48	12.00	3 00	2.66	0 8867	6.21	3	80–90
30/6	8	214	35	4 38	3.679	2.55	0 6931	4 85	7	30–40
15/7	4	83	69	17.25	3 105	1 58	0.5088	1.52	3	30–40
15/	6	120	27	4.50	3.534	6 70	1.8959	9 48	5	90–95
28/7	8	214	35	4.38	3 68	14.23	3 866	9 48	7	90–95
10/9 77	4	198	116	29 0	11 89	2 00	0 1682	0.504	3	5–10
10/9	2	81	48	24 0	9 84	18 00	1.8292	1 83	1	80–90
10/9	2	100	73	36.5	9 96	24.50	2.48	2 48	1	80–90
10/9	2	192	29	14 50	12 33	0.500	0 0406	0 0406	1	10–20
10/9	3	123	75	25.00	9 75	9 080	0 9231	1 846	2	60–70
10/9	3	114	65	21.67	9.32	24 340	2.6111	5.22	2	90–95
20/10	9	638	174	19.33	14 11	11.25	0.7973	6.38	8	40–50
20/10	24	256	68	6 18	2 719	2 23	0 8201	18 86	23	20–30
20/10	25	250	70	2 80	2.016	2.916	1 4464	34 7	24	90–95

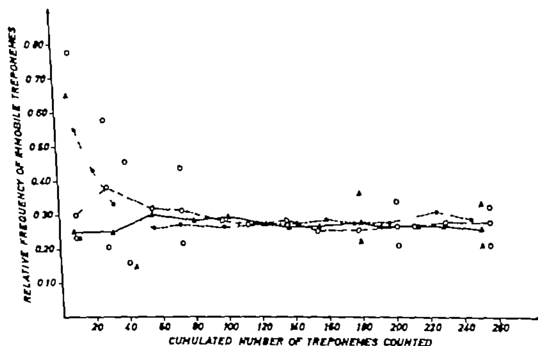


Fig 2 Relative frequencies of immobile treponemes found in continuous counts of organisms in three samples from the same tube. The points outside the drawn graphs represent 95 per cent confidence limits corresponding to the counts.

total of »n« can be predicted from the binomial probability function.

$$p(x = a) = \binom{n}{a} p^{a}(1-p)^{n-a}$$

and that the probability of finding not more than »a« immobile treponemes in »n« is given by the binomial distribution function.

$$P(x \leq a | a, n, p) = \sum_{x=0}^{a} \binom{n}{x} p^{x}(1-p)^{n-x} \quad (A)$$

The agreement between the mathematical model just described and the observations made may also be illustrated by Fig 2 which shows the outcome of counting treponemes in three separate samples from the same tube.

DISCUSSION

The random experiments presented in this paper indicate that the Poisson and binomial distributions are adequate mathematical models for the distribution of both motile and immobile treponemes in suspensions when examined in slide preparations. The randomization of visual fields examined was essential to establish the nature of the distributions. These two mathematical models may thus serve to determine the precision of the total counts of treponemes and the precision of the relative frequencies of immobilized treponemes. The latter depends on the number of treponemes counted and on the probability parameter of the distribution, the binomial variance of the relative frequency being $p(1-p)/n$, where n is the total number of treponemes counted and p the probability parameter of immobile treponemes, this being unknown. The relative frequency determined from observations is the best estimate of p Fig 3 shows the 95 per cent confidence limits of this estimate for counts of a total of 25 50 and 100 treponemes and for relative frequencies ranging from 8 to 96 per cent.

In most laboratories, total numbers of 25 or 50 are counted and the number of immobile treponemes enumerated. Authors like *Magnuson & Thompson* (5) and *Ledbetter & Martens* (4) counted 25 treponemes, while *Nielsen* (9) counted both 25 and 50 The originators, *Nelson & Mayer* (6) counted 50 as did *Harris et al* (3) and *Portney et al* (10), while *Müller & Pop Aceva* counted 25 and sometimes 50 (7)

In the present experiments, counts of 25 50 etc. have not been adhered to If a predetermined number of treponemes are to be counted, the last treponeme included in the count will frequently not be alone in the visual field. It will often be together with some other treponemes which may be those succeeding the number to be counted (*e.g* n treponemes are to be counted, but the numbers n−2, n−1 n, n+1 n+2 etc. are in the visual field, and which one is number n?). This vitiates the precision of the counting by introducing a subjective element

The usual procedure is to incubate the serum to be tested with the same treponeme suspension in two tubes, adding unheated guinea pig serum to the one and heat-inactivated guinea pig serum to the other After incubation, the relative frequency of immobile treponemes in each tube is determined. It is assumed that the only difference between the two samples is that the test tube contains active complement which will immobilize the treponemes if antitreponemal antibody is present in the serum, while the control tube does not contain active complement. The latter tube serves as a control of survival conditions. *If the proportion of immobile treponemes in the control tube is less than that in the test tube, it may be assumed that the two tubes* contain treponemes of two different kinds as regards immobilization. Let a_T and a_C be the number of immobile treponemes in the test tube and the control tube, respectively and n_T and n_C the corresponding total numbers counted. The two relative frequencies a_C/n_C and a_T/n_T can be compared by determining the conditional probability of finding a_T or more immobile treponemes in the test tube, given that the total number of immobile treponemes is $a_T + a_C$, the number of those examined being $n_T + n_C$ Using the hypergeometric distribution.

$$P\{x \geq a_T \mid a_C + a_T\}$$

$$= \sum_{x=a_T} \frac{\binom{n_T}{x}\binom{n_C}{a_C + a_T - x}}{\binom{n_C + n_T}{a_C + a_T}} \quad) \quad (B)$$

This probability can be computed for each pair of testing and control tubes containing aliquots of the same serum and the same treponeme suspension If the conditional probability of finding the number of immobile treponemes observed in the control

) The test is described in *Hald* (1)

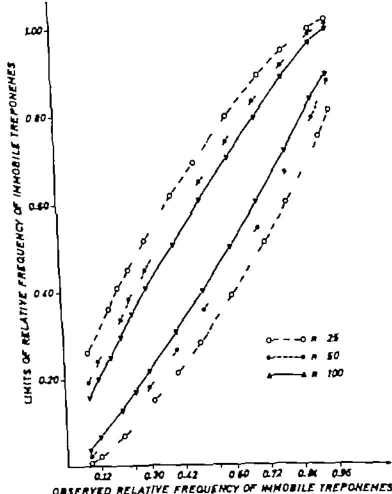


Fig. 3. 95 per cent confidence limits of the estimate of the binomial parameter p in counts of 25, 50 and 100 treponemes. The graphs were constructed from Table IX, *Hald* (1). Linear interpolation was used. This explains the irregularities.

sample is less than a certain predetermined fraction α, the two samples are considered to belong to different treponeme populations as regards immobilization frequency. The fraction α was chosen to be 0.05 because the test is one-sided. Using the probability distribution described by formula (B) a table was constructed showing the conditional probability for $x_C = 0, 1 - - - 6$, $x_T = 5 - - - 14$ and $n_T = n_C = 25$ (Table 6).

On the basis of the figures presented, the following simple rule for evaluation of the outcome of the TPI test is proposed.

1) All sera, the control samples of which contain more than 6 immobile treponemes, should be discarded and new specimens requested.

2) Where fewer than 7 out of 25 treponemes are found to be immobile in the control sample, the test is considered reactive if the probability given in Table 6 is less than 0.05.

In practice, a higher number than 25 treponemes will usually be counted, depending on the number of treponemes in the visual field in which treponeme number 25 is found. This does not affect the proposed criteria materially since the larger number will not restrict the limits proposed.

The data presented above have been obtained by counting treponemes in randomly selected visual fields. This is usually not the case in routine performance of the test. Considerable disagreement has been found between the immobilization percen-

total of n can be predicted from the binomial probability function.

$$p(x = a) = \binom{n}{a} p^a (1-p)^{n-a}$$

and that the probability of finding not more than a immobile treponemes in n is given by the binomial distribution function.

$$P(x \leq a | a, n, p) = \sum_{x=0}^{a} \binom{n}{x} p^x (1-p)^{n-x} \quad (A)$$

The agreement between the mathematical model just described and the observations made may also be illustrated by Fig. 2 which shows the outcome of counting treponemes in three separate samples from the same tube.

DISCUSSION

The random experiments presented in this paper indicate that the Poisson and binomial distributions are adequate mathematical models for the distribution of both motile and immobile treponemes in suspensions when examined in slide preparations. The randomization of visual fields examined was essential to establish the nature of the distributions. These two mathematical models may thus serve to determine the precision of the total counts of treponemes and the precision of the relative frequencies of immobilized treponemes. The latter depends on the number of treponemes counted and on the probability parameter of the distribution, the binomial variance of the relative frequency being $p(1-p)/n$, where n is the total number of treponemes counted and p the probability parameter of immobile treponemes, this being unknown. The relative frequency determined from observations is the best estimate of p Fig 3 shows the 95 per cent confidence limits of this estimate for counts of a total of 25 50 and 100 treponemes and for relative frequencies ranging from 8 to 96 per cent

In most laboratories, total numbers of 25 or 50 are counted and the number of immobile treponemes enumerated. Authors like *Magnuson & Thompson* (5) and *Ledbetter & Martens* (4) counted 25 treponemes, while *Nielsen* (9) counted both 25 and 50 The originators, *Nelson & Mayer* (6) counted 50 as did *Harris et al* (3) and *Portnoy et al* (10), while *Müller & Pop-Aceva* counted 25 and sometimes 50 (7)

In the present experiments, counts of 25 50 etc. have not been adhered to. If a predetermined number of treponemes are to be counted, the last treponeme included in the count will frequently not be alone in the visual field. It will often be together with some other treponemes which may be those succeeding the number to be counted (*e.g.* n treponemes are to be counted, but the numbers n−2 n−1 n, n+1 n+2 etc. are in the visual field, and which one is number n?) This vitiates the precision of the counting by introducing a subjective element.

The usual procedure is to incubate the serum to be tested with the same treponeme suspension in two tubes, adding unheated guinea pig serum to the one and heat inactivated guinea pig serum to the other After incubation, the relative frequency of immobile treponemes in each tube is determined. It is assumed that the only difference between the two samples is that the test tube contains active complement which will immobilize the treponemes if antitreponemal antibody is present in the serum, while the control tube does not contain active complement. The latter tube serves as a control of survival conditions. If the proportion of immobile treponemes in the control tube is less than that in the test tube, it may be assumed that the two tubes contain treponemes of two different kinds as regards immobilization. Let a_T and a_C be the number of immobile treponemes in the test tube and the control tube, respectively and n_T and n_C the corresponding total numbers counted. The two relative frequencies a_C/n_C and a_T/n_T can be compared by determining the conditional probability of finding a_T or more immobile treponemes in the test tube, given that the total number of immobile treponemes is $a_T + a_C$, the number of those examined being $n_T + n_C$ Using the hypergeometric distribution.

$$P\{x \geq a_T | a_C + a_T\}$$

$$= \sum_{x=a_T} \frac{\binom{n_T}{x}\binom{n_C}{a_C + a_T - x}}{\binom{n_C + n_T}{a_C + a_T}} \quad ^{1)} \ (B)$$

This probability can be computed for each pair of testing and control tubes containing aliquots of the same serum and the same treponeme suspension. If the conditional probability of finding the number of immobile treponemes observed in the control

1) The test is described in *Hald* (1)

Acta path microbiol scand Sect. B, 86 275–281 1978.

LIBERATION OF ENDOTOXIN DURING GROWTH OF *NEISSERIA MENINGITIDIS* IN A CHEMICALLY DEFINED MEDIUM

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Andersen, B M & Solberg, O Liberation of endotoxin during growth of *Neisseria meningitidis* in a chemically-defined medium Acta path microbiol scand Sect. B, 86 275–281 1978

Modified medium 199 is well suited to the growth of meningococci if rich growth is not necessary The bacteria were very sensitive to changes in pH and needed a good buffer capacity of the medium Four strains of *Neisseria meningitidis* were studied After the stationary phase had been reached, the viable cell count decreased relatively slowly However the total cell count remained unchanged for at least 12 hours The standardized *E coli* endotoxin could be detected easily by the *Limulus* lysate test in different protein-free, pH-adjusted and ion-balanced liquids, and the determination of endotoxin was reproducible The *Limulus* lysate test was more sensitive than the rabbit pyrogen test as regards meningococcal endotoxin Liberation of endotoxin during growth in modified medium 199 varied with strain and growth conditions Repeated investigations showed the same pattern of growth and endotoxin liberation for each strain, also under other growth conditions When sonicating the bacteria, high yields of endotoxin were obtained from each strain

Key words *Neisseria meningitidis*, free endotoxin

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Endotoxin may play a central role in the pathogenesis and production of disease manifestations of meningococcal infections (5 8 9 21 24). Endotoxin activates vasoactive amines and induces intravascular and peripheral circulatory collapse (8) Even if *Neisseria meningitidis* has less endotoxin than *Escherichia coli* or *Salmonella typhimurium*, its toxin is five to ten times more active in terms of inducing the dermal Shwartzman reaction (5)

Besides the classical rabbit pyrogen test, endotoxin can be detected by various other methods (5 6 18 22) The *Limulus* lysate test is semiquantitative (18) and quite satisfactory as compared to the rabbit pyrogen test, if proper adjustment of pH and ion content is made and attention is paid to the inhibitory effect of protein (2 4 11 15)

The purpose of this investigation was to study the growth conditions and liberation of endotoxin from *N meningitidis* during growth in a chemically defined, protein-free medium.

MATERIAL AND METHODS

Bacterial strains Four isolates were studied, three from patients with meningitis and one from a healthy carrier (strain 247) Details of serogroups and resistance to antibiotics are shown in Table 1 The isolates were typical meningococci in terms of morphology growth and acid production from sugars. The strains were received from the Department of Microbiology Ullevaal Hospital The serogrouping was kindly verified by *E Holten*, Oslo After isolation, the strains had been subcultivated on heated blood agar four or more times before investigation

TABLE 6 *The Conditional Probability of Finding a_T or more Immobile Treponemes in the Test Tube Given that the Total Number of Immobile Treponemes is $a_T + a_C$, the Number of those Examined being 25 for both Tubes*

a_C / a_T	0	1	2	3	4	5	6
5	0.225	0.095	0.209	0.351	0.500	0.637	0.752
6	0.011	0.049	0.123	0.232	0.363	0.500	0.629
7	$0.0^{2}48$	0.024	0.069	0.145	0.248	0.371	0.500
8	$0.0^{2}20$	0.011	0.037	0.085	0.160	0.260	0.370
9	$0.0^{3}82$	$0.0^{2}58$	0.0186	0.048	0.098	0.173	0.269
10	$0.0^{3}32$	$0.0^{2}22$	$0.0^{2}90$	0.025	0.057	0.108	0.182
11	$0.0^{3}12$	$0.0^{3}96$	$0.0^{2}42$	0.012	0.031	0.064	0.116
12	$0.0^{4}43$	$0.0^{3}38$	$0.0^{2}18$	$0.0^{2}60$	0.016	0.036	0.070
13	$0.0^{4}15$	$0.0^{3}14$	$0.0^{3}74$	$0.0^{2}27$	$0.0^{2}78$	0.019	0.040
14	$0.0^{5}48$	$0.0^{4}51$	$0.0^{3}29$	$0.0^{2}11$	$0.0^{2}35$	$0.0^{2}93$	0.021

tages found using the routine procedure and the random technique described when the proportion of immobile treponemes occurred in the range from 20 to 75 per cent.

The limits of error derived from the two mathematical models only concern the exactness of the counting other factors may seriously influence the outcome of the test. Two of these were investigated. 1) The sojourn of the mixture of treponemal suspension, syphilitic serum and complement outside the incubator in the atmosphere of the laboratory and 2) exposure of the serum and complement mixtures to treponemal suspensions harvested from different animals.

The result of these investigations will be published in a subsequent paper

I am indebted to *M Weis Bentzon*, Biostatistical Department, for his criticism, and I am grateful to *B B Jørgensen* and the TPI staff Treponematoses Department, Statens Seruminstitut

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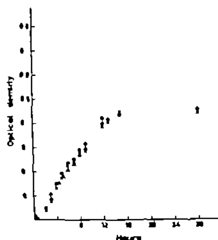


Fig. 1. Four different growth curves for strain 247 in modified medium 199 at 37° C in 5% CO_2. Each curve is the mean of two or three parallels.

Growth in medium. The bacteria were sensitive to changes in pH and grew well only in buffered medium. The CO_2 enhanced the growth but at the same time adversely acidified the medium. The buffer capacity of the medium was evaluated by phenol red during growth (colour change to yellow at pH $\leq$ 6.8). The change of colour had no effect on the spectrophotometric recordings. The growth pattern of each strain was reproducible, as shown in Fig. 1. All strains had different growth curves, but the differences between them were very small (Fig. 2, a, b, c, d). The exponential phase lasted between 8–14 hours, when the bacterial count was increased from 5×10^7 to 10^9 for the four strains. The corresponding increase in optical density was between ½ and 1 logarithmic unit. After the stationary phase had been reached, the total cell count remained unchanged for at least 12 hours, while the vital cell count decreased relatively slowly. This was most evident with strain 270 (Fig. 2 b).

Fig. 2. Growth curves for strains 247 (Fig. 2 a), 270 (Fig. 2 b), 714 (Fig. 2 c), and 840 (Fig. 2 d). O——O = total bacterial count, ●——● = viable count, △——△ = optical density.

TABLE 1 *Meningococcal Isolates, Serogroup and Antibiotic Resistance*

Isolate	Isolated from	Serogroup	Antibiotic resistance[a] S	P	C	A	E
247	Pharynx	Y	4	1	2	1	1
270	CSF	B	4	1	2	1	2
714	CSF	A	4	1	2	1	1
840	CSF	B	4	1	2	1	1

[a] Discs from AB Biodisk on lysed blood agar in 5% CO_2 S = sulphonamide, P = benzylpenicillin, C = chloramphenicol, E = erythromycin. A = ampicillin. 1 = sensitive, 2 = fairly sensitive 4 = resistant.

Medium. 9 867 g dehydrated medium number 199 (16) with Earle's unmodified salts without sodium bicarbonate ($NaHCO_3$), 3 g dextrose, 0 2 mg cocarboxylase and 2 g disodium hydrogen phosphate (Na_2HPO_4 12 H_2O) were dissolved in sterile pyrogen free water to 1000 ml. The pH was adjusted to 7 5 with 1M NaOH before filtration through a pyrogen-free prefilter (Gelman Metrigard, superfine) and membrane filter (Gelman, Metricel, G A. 8 0 2 µm) The pH after filtration was between 7 4 and 7.5

Optical density (OD) Optical density was measured by Beckman Model DU 2 Ultraviolet Spectrophotometer wave length 620 nm and slit 0 04 mm Belco nephelo culture flasks (300 ml) with a cylindric side arm fitted to an adaptor in the spectrophotometer were used.

Before growth in liquid medium the meningococci were grown for 18 hours on heated blood agar at 37° C in 5 per cent CO_2 and harvested with a flamed platinum loop. Precultures were washed in sterile, pyrogen-free saline (0 9 per cent NaCl) three times by high speed ultracentrifugation (Sorvall) 9770 × g at 20° C. It was ascertained that all equipment was pyrogen-free, and all procedures were carried out aseptically The inoculate was adjusted by saline to an OD of 0 6 equivalent to a colony count of 10^8–10^9 per ml One ml of inoculate was transferred to 40 ml growth medium 199 There was no apparent opacity of the medium after inoculation. Vigorous agitation was applied to the growth flasks before measuring the OD Two to three parallel flasks were used for each growth curve.

Total count The total number of bacteria was determined by Burker's chamber If a tendency to spontaneous agglomeration occurred, agitation on a Rotamixer was first applied in order to disperse the cells to facilitate counting.

Viable count Aliquots of 0 1 ml were withdrawn from the culture flasks for viable counts The culture was filtered through sterile, non-toxic filters (Extension set with final filter Travenol code 2CO240) with pore size 0 45 µm The filters allowed 0–1 bacteria to pass through when 5 ml of a culture containing 10^8–10^9 bacteria/ml was filtered The filtrate was stored at −20° C before examination for endotoxin

Endotoxin Determination

a) *Limulus lysate test* In the presence of endotoxin, *Limulus amoebocyte* lysate is transferred from liquid to a gel. *Limulus* lysate was diluted with pyrogen-free water as described by the producer *E coli* endotoxin was diluted and mixed well for each dilution stepwise down to 0 1 ng/ml which was the lower sensitivity limit of the *Limulus* lysate test *E coli* endotoxin was used as positive control.

The tube method was compared with a glass slide procedure (7) and the two were found to be equally sensitive. Endpoint was easier to determine by the slide method, which also required less *Limulus* reagent (10 µl) as compared to the tube method (0 1 ml). The test could be read after a shorter period (viz. 30 minutes) than the tube technique Before use, the reagents and samples were heated to 37° C. All solutions were mixed well immediately before use. Glass slides were placed in moist chambers to prevent evaporation (a large Petri dish with moist blotting-paper covering the bottom) and 3 drops of 10 µl *Limulus* lysate reagent were placed on the slides with a sterile pipette (Oxford pipette) Ten µl of the solution to be tested was applied to the drop of *Limulus* lysate and both were mixed well with the tip of the pipette. The moist chamber was incubated at 37° C

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b) *Rabbit pyrogen test* was carried out according to Pharmacopoea Nordica (20) As positive test was used the sum of maximum temperature increase greater than 2 65 C in three rabbits during three hours after intravenous injection of pyrogenous material Because of shortage of material, this test was not always performed on all three rabbits

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RESULTS

Morphology The colonies of all *N meningitidis* strains had smooth glistening surfaces, and were 1–3 mm in diameter on heated blood agar After passage in the liquid medium 199 strain 247 showed a few rough colonies which disappeared on second passage on agar

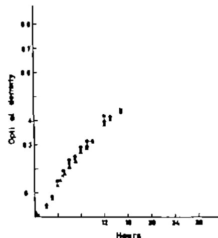


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TABLE 5 *Meningococcal Endotoxin. Limulus Lysate Test Compared with Rabbit Pyrogen Test*

Dilution	Strain 840 R	840 L	247 R	247 L	270 R	270 L	714 R	714 L
1/10	+	+	+	+	–	–	–	–
1/30	+	+	–	+				
1/100	+	+	–	+				
1/200	+	+	–	+				
1/500	+	+	–	–				
1/1000	–	+						
1/2000	–	+						
1/4000	–	+						
1/8000	–	–						

R = rabbit pyrogen test, L = *Limulus* lysate test, x) Meningococcal endotoxin in filtrate from 20–24 hour cultures

after the stationary phase was reached. Repeated investigations showed the same pattern of growth and endotoxin liberation for each strain (Table 4) Under other growth conditions, on addition of 0 02 g $FeCl_3$, 0 1 g L-glutamine, 2.5 mg NAD (V factor) 1 0 mg hemine (X factor) and 0 1 mg vitamin B_{12} to 1000 ml medium 199 strain 840 still liberated endotoxin and strain 270 did not. When the bacteria were sonicated, high yields of endotoxin were obtained from each strain.

Rabbit pyrogen test The *Limulus* lysate test was compared with the rabbit pyrogen test The culture filtrates with maximum endotoxin content from strains 247 and 840 and filtrates taken approximately at the same growth phase from the two strains which were endotoxin negative by the *Limulus* lysate test, were tested in different dilutions on rabbits

As shown in Table 5 the *Limulus* lysate test was more sensitive for meningococcal endotoxin than the rabbit pyrogen test, both as regards samples with high and low endotoxin content.

DISCUSSION

Medium There is considerable variation between different *N meningitidis* strains, both as regards growth factor requirement and virulence (1 13 14) Each of the four strains studied in this investigation showed their own nutritional needs and varied in endotoxin liberation. The chemically-defined, protein-free liquid medium 199 was chosen, since it gave good support to growth of *N meningitidis*, and since protein adversely influences the *Limulus* lysate test (2 4)

During exponential growth, the bacteria are more vulnerable to osmotic or other changes which can induce autolysis (12) The observation that the total cell count did not decrease during the first 12 hours of the stationary phase indicates that the growth medium was osmotically optimal.

With the exception of strain 270 the viable count was nearly the same after 24 and 28 hours Thus, the endotoxin in the culture filtrates, was probably liberated mainly from whole living or dead non-disintegrated bacterial cells.

Endotoxin determination *Dawson et al* (4) determined the endotoxin content in meningococcal polysaccharide vaccines by both the rabbit pyrogen test and the *Limulus* lysate test and good correlation was found between the two methods. The *Limulus* lysate test has also been sensitive to meningococcal endotoxin in cerebrospinal fluid and other body fluids with a low protein content (17 23) The lysate test was more sensitive to meningococcal endotoxin than the rabbit pyrogen test. This is in accordance with what has been found for other bacteria endotoxins (19) However the endotoxin toxicity in rabbits is dose-dependent, whereas the *Limulus* test is dependent on the concentration of endotoxin at a limited observation period (2).

Endotoxin liberation from *N meningitidis*. *Zollinger et al.* (25) showed that outer cell wall complex could be isolated from the medium, from supernatant filtered through 0 45 μm Millipore filter and from whole *N meningitidis* cells. They found that the relative amount in cell wall substances with endotoxic effect varied from one strain to another Endotoxin was liberated from three of our strains, while one, strain 270 did not liberate endotoxin though under the same growth conditions. This strain still did not liberate endotoxin under other growth conditions, while strain 840 liberated only moderate amounts.

DeVoe & Gilchrist (6) have shown that endotoxin is liberated during the exponential growth phase Up

TABLE 2 *Sensitivity of Limulus Lysate Test to Endotoxin in Different Sterile Liquids*

Endotoxin: *E coli*	Sterile water	NaCl	Ringer's acetate	Medium 199	Plasma (human)
100 μg/ml	+	+	+	+	+
10 μg/ml	+	+	+	+	–
1 μg/ml	+	+	+	+	–
100 ng/ml	+	+	+	+	–
10 ng/ml	+	+	+	+	–
1 ng/ml	±	+	+	+	–
0 1 ng/ml	–	–	±	±	–
0 01 ng/ml	–	–	–	–	–

+ = gelatinization, – = no gelatinization, ± = traces of endotoxin

TABLE 3 *Meningococcal Endotoxin in Filtrates from Cultures Determined by the* Limulus *Lysate Test*

Isolate	Endotoxin dilution after growth						
	Hours						
	0	3	5	8	13	19	24
247	–	1/10	1/10	1/100	1/100		1/200
270	–	–	–	–	–	–	–
714	–	–	–	–	–	–	–
840	–	1/400		1/1000	1/2000	1/4000	

– = no detectable endotoxin Endotoxin content in the inoculate which started the cultures. 247 1/100 270 – 714 1/1 840 1/100

Endotoxin Investigation

Limulus *lysate test* The sensitivity of the *Limulus* lysate test was compared in different liquids (Table 2) The standardized *E coli* endotoxin could be detected easily in different protein-free, pH-adjusted and ion balanced liquids such as Ringer's acetate, 0 9 per cent NaCl sterile water and medium 199 However detection of endotoxin was difficult in small samples of undiluted plasma, regardless of filtration through Millipore filters (0 45 μm), or heating to 56° C for 30 minutes.

No free endotoxin was detected in filtrates from strain 270 and only traces from strain 714 (Table 3) Strain 247 liberated a small amount and strain 840 more copious amounts of endotoxin. Maximum endotoxin liberation occurred a few hours

TABLE 4 *Liberation of Meningococcal Endotoxin in Filtrates from Culture of Strain 840*

Isolate	Hours					
840	0	3	8	13	19	24
1	–	1/400	1/1000	1/2000	1/4000	
	–	1/400	1/1000	1/2000	1/4000	
	–	1/400	1/1000	1/2000	1/4000	
2	1/1	1/10	1/100	1/100		1/1000
	±	1/10	1/1000	1/1000		1/1000
3	–	1/1	1/100	1/1000		1/1000
	–	1/1	1/10	1/100		1 /1000

– = no detectable endotoxin, ± = traces of endotoxin. 1 2, 3 = three times repeated studies, each in two or three parallel growths.

Brandon, N. Duggan, M. Oomen, J. M. V. & de Rajkovic A. D. Meningococcal antigen in diagnosis and treatment of group A meningococcal infections. Amer. J. Med. 58 823–828, 1975

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to 18% of the total content of endotoxin, determined on the basis of KDO (2 keto-3-deoxyoctonate) was derived from vesicles in the outer cell wall. Thus liberation of endotoxin from meningococci during growth may stem from soluble cell wall complexes possibly liberated as vesicles (3 6) Also non-pathogenic strains of *Neisseria* grown in simple, defined media are shown to liberate different amounts of free lipopolysaccharides (10)

The fact that one of the four strains in our investigation did not liberate a detectable amount of endotoxin (although the bacteria were shown to have endotoxin in cell walls when sonicated) might be due to loss of the ability to liberate endotoxin on subcultivation. Since the four strains were handled in the same way it is likely that there was an inherent difference in the outer cell wall layer or strains. It is impossible to relate this liberation of endotoxin to specific serogroups since one serogroup B delivered large amounts of free endotoxin while the other did not liberate filtrable endotoxin at all.

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Acta path. microbiol. scand. Sect. B, 86: 283–290, 1978

IDENTIFICATION OF *SALMONELLA* BACTERIA BY CO-AGGLUTINATION, USING ANTIBODIES AGAINST SYNTHETIC DISACCHARIDE-PROTEIN ANTIGENS O2, O4 AND O9, ADSORBED TO PROTEIN A-CONTAINING STAPHYLOCOCCI

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Svenungsson, B. & Lindberg, A. A. Identification of *Salmonella* bacteria by co-agglutination, using antibodies against synthetic disaccharide-protein antigens O2, O4 and O9, adsorbed to protein A containing staphylococci. Acta path. microbiol. scand. Sect. B, 86: 283–290, 1978.

Protein A-containing staphylococci sensitized with antisera against synthetic *Salmonella* O-antigens 2, 4 and 9, representative of serogroups A, B and D, respectively, were used for identification of *Salmonella* bacteria by co-agglutination. Out of 416 *Salmonella* bacteria tested the reagents correctly identified all 24 serogroup A strains, 119 serogroup B strains and 39 serogroup D strains. Unexpected agglutination was registered with two of 144 strains belonging to serogroup C 2 with reagent containing antiserum against synthetic O antigen 4. No agglutination occurred when 24 non-*Salmonella* bacterial strains were tested. Approximately 10^8 bacteria were required for positive co-agglutination. As compared to standard slide agglutination with conventional anti-*Salmonella* O factor sera, the co-agglutination method was favourable in that the reactions were stronger, although the concentration of antiserum used was from 20 to 200 times lower. The co-agglutination method could also be used for detection of soluble antigens in the form of lipopolysaccharides from *Salmonella* bacteria in concentrations of 1 µg/ml. When the sensitivity of the co-agglutination technique was compared with indirect immunofluorescence (IFL), the IFL method was shown to be at least 1000 times more sensitive.

Key words: *Salmonella* identification; co-agglutination; synthetic disaccharide-protein antigens.

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The accuracy in identification of *Salmonella* bacteria by immunological methods is dependent on the specificity of the antibody preparations used. Conventional procedures for preparation of monospecific antisera against *Salmonella* O-antigens, which define the serogroup specificity, involve immunization of rabbits with whole heat- or formalin-killed bacteria, followed, in most instances, by absorption of serum with related bacteria for removal of cross-reacting antibodies (10, 19). However, the absorptions are often incomplete and the titre of the desired antibodies is also often decreased. Moreover the absorption is a time consuming procedure.

The structure of the O-antigenic polysaccharide chain in the lipopolysaccharide (LPS) from *Salmonella* serogroups A, B and D, representing about 70% of all *Salmonella* bacteria isolated in humans (28), has been determined previously (13–16, 23). The immunodominant group-specific O-antigenic determinants in these serogroups can be represented by disaccharides, which are part of the tetrasaccharide repeating units making up the O-antigenic

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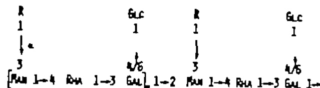


Fig 1 Structure of the O-antigenic polysaccharide chain in the lipopolysaccharide from *S paratyphi* A var durazzo serogroup A *S typhimurium* serogroup B and *S enteritidis* serogroup D according to *Hellerqvist et al.* (14–16). R is paratose in serogroup A abequose in serogroup B and tyvelose in serogroup D

Abbreviations used. Gal *D*-galactose
Glc, *D*-glucose
Man, *D*-mannose
Rha, *L* rhamnose
n = 5–30

polysaccharide chain. In serogroup A, B and D the disaccharides paratose 1→3 mannose, abequose 1→3mannose and tyvelose 1→3 mannose represent the O-antigens 2 4 and 9 respectively (Fig 1). These antigenic determinants have been synthesized and linked to bovine serum albumin (BSA) as an immunogenic carrier (1 2, 11 12 24) Antisera from rabbits immunized with the synthetic disaccharide-protein conjugates were shown to be highly specific when tested in quantitative precipitation, passive haemagglutination enzyme-linked immunosorbent assay (ELISA) and complement mediated bactericidal tests (4 11 18). We also demonstrated the high specificity of these antisera when used in indirect immunofluorescence for identification of *Salmonella* bacteria (26 27).

The co-agglutination (COA) method introduced by *Kronvall* (20) is a simple and rapid method for serological grouping of bacteria (6–9 20 25) The reagent consists of *Staphylococcus aureus* bacteria containing protein A which reacts with the Fc part of immunoglobulin G (IgG) This leaves the antigenic binding site of IgG free for interaction with antigen.

The present paper deals with the use of the antisera against synthetic disaccharide-protein antigens for sensitization of protein A-containing staphylococci for identification of *Salmonella* bacteria by COA. The method was also compared with the standard slide agglutination test (SSA) and its sensitivity compared with that of the indirect immunofluorescence test (IFL)

MATERIAL AND METHODS

Bacterial strains and cultivation methods The enterobacterial strains used came from the culture collection at the Department of Bacteriology National Bacteriological Laboratory Stockholm, Sweden, and from fresh faecal samples delivered to the laboratory for examination of pathogenic enteric bacteria.

Isolation and identification of the bacteria were performed as described elsewhere (10 19) *Salmonella* bacteria were isolated on desoxycholate-citrate agar plates (Dc-agar Oxoid CM 35) and on brilliant green-phenol red agar plates (Bg-agar) after enrichment in Rappaport s broth. Some of the *Salmonella* bacteria were also grown on blood agar base (BA, no 2 Oxoid CM 271) and endo-agar plates. For comparison of the sensitivity of COA with the sensitivity of IFL, the bacteria were diluted in phosphate buffered saline (PBS) (pH 7 2) The concentration range was from 2×10^9 to 2×10^4 bacteria/ml in tenfold dilution steps.

Staphylococcus aureus strain Cowan I, known to produce large amounts of protein A, was used for preparation of COA reagents. The strain came from Professor *Tord Holme* Department of Bacteriology Karolinska institutet, Stockholm, Sweden.

Lipopolysaccharide extraction Lipopolysaccharides (LPS) were extracted from *S paratyphi* A var durazzo (O-antigen 2 12_1 12_3), *S typhimurium* strain SH4809 (O-antigen 4 5 12_2) and *S enteritidis* strain SH1262 (O-antigen 9 12_2) as described previously (22).

Synthetic immunogens and immunization procedure The synthesis of the immunogens methyl 3-*O*-(α-paratopyranosyl)-α-D-mannopyranoside, methyl 3-*O*-(α-abequopyranosyl)-α-D-mannopyranoside and *p*-isothiocyanatophenyl 3-*O*-(α-tyvelopyranosyl)-α D-mannopyranoside, representing *Salmonella* O-antigens 2, 4 and 9 respectively has been described previously (1 2, 12) The immunogens were coupled to bovine serum albumin (BSA) via the synthesis of the phenylisothiocyanate disaccharide glycoside, as described previously (11 24) In this investigation the immunogens will be referred to as PM BSA (O2-specificity), AM BSA (O4-specificity) and TM BSA (O9-specificity) Immunization of rabbits with the synthetic disaccharide-protein conjugates was performed as described previously (26 27). Quantitative antibody determinations using ELISA (3 5) with the various lipopolysaccharides and BSA as antigens, showed that more than half of the antibody specificity in the PM BSA, AM BSA and TM BSA antisera was directed against the disaccharide hapten as compared to BSA (data not shown). *Salmonella* O factor sera for SSA tests were prepared according to *Kauffmann* (19)

Preparation of protein A-containing staphylococci Preparation of the staphylococci was performed essentially as described by *Kronvall* (20) The bacteria were grown overnight at 37°C in Trypticase soy broth (TSB BBL 11768). The suspension was centrifuged (3000 *g* + 3°C 15 min), the supernatant discarded and the pellet resuspended in PBS This washing procedure was repeated three times. The bacteria were then treated with 0.5% formaldehyde for three hours at room temperature. After three additional washing procedures in PBS, the bacteria were resuspended to 10% (v/v) in PBS Following heat treatment at 80°C for ten minutes, the washing procedure was repeated twice and the bacteria were finally resuspended to 10% as before. The bacteria were then stored at 4°C until used (usually within two

weeks) A 10% suspension of staphylococci prepared in this way was shown to bind approximately 0.4 mg of IgG/ml as estimated essentially by quantitative methods described elsewhere (21) (The estimation was performed at Pharmacia Diagnostics, Uppsala, Sweden).

Preparation of sensitized staphylococci 0.1 ml of antiserum was added to 1 ml of the 10% formaldehyde and heat-treated staphylococcal suspension. The mixture was kept at room temperature for three hours under continuous agitation. The suspension was centrifuged (3000 g, +3°C, 15 min) and the sensitized bacteria were washed twice in PBS and subsequently resuspended to 1% (v/v) and 2% (v/v) in PBS. The reagent was stored at 4°C until used (usually within two weeks).

Co-agglutination (COA) test A few colonies of the bacterial strains, or 0.05 ml of the bacterial suspension to be investigated, were suspended in 0.05 ml of COA reagent on a glass slide, which was rocked back and forth for two minutes. Agglutination was registered during this period and was recorded as 2+ when clear by the naked eye, and as 1+ if a magnifying glass was needed for observation.

Indirect immunofluorescence (IFL) test The indirect immunofluorescence method was used as described previously (26). The anti PM-BSA, AM-BSA and TM-BSA sera were diluted 1/160, 1/80 and 1/160 respectively. The fluoresceinisothiocyanate conjugated sheep anti rabbit globulin, molar F/P ratio 4.6, was obtained from the Department of Immunology, National Bacteriological Laboratory, Stockholm, Sweden. The working titre of the conjugate was 1/40. A Leitz orthoplan fluorescence microscope was used with incident light and a HBO-200 mercury lamp as light source and a magnification of 750×. The fluorescence reactions were estimated as described earlier (26).

RESULTS

Co-agglutination (COA) Compared with Standard Slide Agglutination (SSA) with Known Salmonella *Bacteria*

Staphylococcus aureus Cowan I bacteria were sensitized with various dilutions of sera against PM-BSA (O2), AM-BSA (O4) and TM-BSA (O9). The reagents were then tested against indicator strains to determine a suitable working dilution for each serum. The indicator strains used were *S. paratyphi* A (O-antigen 2, 12), *S. typhimurium* (O-antigen 4, 5, 12) and *S. enteritidis* (O-antigen 9, 12). The endpoint titre of the three sera used, defined as the highest dilution which gave a 2+ agglutination, was 1/10 (corresponding to a final serum dilution of 1/1000) when the sensitized staphylococci were suspended to 1% (v/v) in PBS and 1/20 (corresponding to a final serum dilution of 1/1000) when suspended to 2% (v/v) (Table 1). When the reagents were tested against the heterologous indicator bacteria, no agglutination occurred at any serum dilution. Nor did unsensitized staphylococci give any agglutination with the indicator bacteria. A 1% suspension of staphylococci sensitized with serum diluted 1/5 (all three anti-sera) was used in most of the subsequent experiments. Routinely prepared *Salmonella* O factor sera 2, 4 and 9 for the SSA were used in dilutions 1/5, 1/20 and 1/50, respectively.

A total of 124 *Salmonella* bacteria were tested against the three reagents. The strains represented *Salmonella* serogroup A (O1, 2, 12), B (O1, 4, 5

TABLE 1. Co-agglutination (COA) of Salmonella *Bacteria with Staphylococci Sensitized with Different Dilutions of Antisera against PM-BSA, AM-BSA and TM-BSA*

Reagent containing antiserum against	Serum dilutions used for sensitization				
	UD[a]	1/5	1/10	1/20	1/40
PM-BSA[b]	2+(15)	2+(20)	2+(120)	–	–
AM-BSA[b]	2+(1)	2+(15)	2+(120)	–	–
TM-BSA[b]	2+(15)	2+(20)	2+(120)	–	–
PM-BSA[c]	2+(1)	2+(15)	2+(40)	2+(90)	–
AM-BSA[c]	2+(1)	2+(15)	2+(40)	2+(90)	–
TM-BSA[c]	2+(1)	2+(15)	2+(40)	2+(90)	–

The PM-BSA reagent was tested against a *S. paratyphi* A strain
The AM-BSA *S. typhimurium* strain
The TM-BSA *S. enteritidis* strain
a) undiluted
b) sensitized staphylococci suspended to 1% in PBS
c) sensitized staphylococci suspended to 2% in PBS
The number of seconds for agglutination clearly visible to the naked eye to occur is shown in brackets.

TABLE 2. *Co-agglutination (COA) Compared with Standard Slide Agglutination (SSA) of Known Salmonella Bacteria For the Co-agglutination Test Staphylococci Sensitized with Anti PM BSA Anti AM BSA and Anti TM BSA Sera were Used For the Standard Slide Agglutination Test* Salmonella *O Factor Sera 2 4 and 9 were Used*

Salmonella serogroup	No of strains	No positive in COA[a] with anti												No positive in SSA[a] with anti											
		PM BSA				AM BSA				TM BSA				O2				O4				O9			
		E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc
A (O1,2,12)	24	24	24	24	24	0				0				22	8	23	9	0				0			
B (O1 4,5 12)	25	0				25	25	25	25	0				0				25	25	25	25	0			
C (O6 7 6 8 8,20)	25	0				1				0				0				0				0			
D (O1 9 12)	25	0				0				25	25	25	22	0				0				25	25	25	24
E (O3 10,3 15; 3,34 1,3 19)	25	0				0				0				0				0				0			

[a]) 2 + reactions

E = endo-agar Bg = brilliant green-phenol red agar
BA = blood agar base Dc = desoxycholate-citrate agar

No entry = reaction not determined

12) C (O6 7 6 8 8 20), D (O1 9 12), and E (O3 10 3 15 3 34 1 3 19) Each strain was grown on four different substrates. blood agar base (BA), endo-agar desoxycholate-citrate agar (Dc-agar) and brilliant green-phenol red agar (Bg-agar). In COA, all serogroups A B and D bacteria gave a strong and direct agglutination (within seconds) with the homologous reagent, irrespective of whether the bacteria were grown on BA endo-agar or Bg-agar (Table 2) When colonies from Dc-agar were tested, the agglutination was more difficult to record, but was still positive. None of the strains gave a positive reaction with the heterologous reagent (tested only with colonies grown on endo-agar). Strains representing serogroups C and E did not cause agglutination, with one exception. One of 14 strains belonging to serogroup C2 agglutinated with the anti AM BSA sensitized reagent (Table 2) However the agglutination could not be seen until after 1 min.

When SSA with factor O2, O4 and O9 sera were used, the reactions were comparable with COA as regards bacteria belonging to serogroups B and D (Table 2). Of the serogroup A bacteria grown on endo-agar 22 out of 24 agglutinated in the factor O2 serum, the agglutination was relatively weak, although visible to the naked eye. In many instances no agglutination was seen with colonies grown on the other substrates. No agglutination occurred when bacteria belonging to serogroups C and E were tested (Table 2)

Co-agglutination of Unknown Bacteria from Faecal Samples

The applicability of the COA method for typing *Salmonella* bacteria was subsequently investigated by testing unknown enteric bacteria from fresh faecal samples. Most of the bacterial colonies tested were taken from Bg-agar or endo-agar A total of 316 bacterial strains were tested and also identified by conventional methods. All 94 *Salmonella* serogroup B strains and 14 serogroup D strains gave a strong and direct agglutination in homologous reagents (Table 3) No false positive reactions were observed. Out of 184 strains representing *Salmonella* serogroups C1 (O6 7) C2 (O6 8), E1 (O3 10) E4 (O1 3 19) G2 (O1 13 23), K (O18) and L (O21) positive reaction was observed with one strain belonging to serogroup C2 with the anti AM BSA sensitized reagent. No positive reactions were recorded among the 24 non-*Salmonella* strains (Table 3). Strains belonging to the genus *Proteus* dominated in this group No *Salmonella* serogroup

TABLE 3. *Co-agglutination (COA) of Suspect Salmonella Bacteria*

Serogroup	No of strains	No. of positive in COA*) with anti PM BSA	AM BSA	TM BSA
B (O1 4,5 12) 15 species	94	0	94	0
C1 (O6,7) 1 species	22	0	0	0
C2 (O6 8) 4 species	130	0	1	0
D1 (O1 9 12) 4 species	14	0	0	14
E1 (O3 10) 5 species	20	0	0	0
E4 (O1,3 19) 2 species	9	0	0	0
G2 (O1 13,23) 1 species	1	0	0	0
K (O18) 1 species	1	0	0	0
L (O21) 1 species	1	0	0	0
Non Salmonella	24	0	0	0
Total	316	0	95	14

a) 2+ reactions were recorded

A bacteria were found among the samples examined.

Co-agglutination of Lipopolysaccharides from Salmonella Bacteria

One advantage of the COA method as compared to SSA is the ability to detect soluble antigens (20 25). Lipopolysaccharides (LPS) extracted from *S paratyphi* A var durazzo (O2, 12_1 12_3), *S.typhimurium* SH4809 (O4 5 12_2) and *S enteritidis* SH1262 (O9 12_2) were suspended in PBS in tenfold dilution steps in concentrations ranging from 10^3 μg/ml to 10^{-2}μg/ml. 0 05 ml of each suspension was added to 0 05 ml of COA reagents as before. Agglutination in homologous reagents occurred in concentrations from 1 μg/ml and upwards. No agglutination was seen when lipopoly saccharides in concentrations of up to 1000 μg/ml were tested against heterologous reagents (Table 4). No agglutination appeared when unsensitized staphylococci were used. It should be stressed that the agglutination reactions were evident but not as strong as when whole bacteria were tested. Further more, the agglutination did not appear until after 90 seconds, although undiluted serum was used to sensitize the staphylococci.

Sensitivity of the Co-agglutination Method Compared with that of Indirect Immunofluorescence

A further investigation was carried out for the purpose of comparing the sensitivity of the COA method with that of indirect immunofluorescence. Overnight cultures in nutrient broth (Difco, Bacto no 3) of the indicator bacteria (*S paratyphi* A, *S typhimurium* and *S enteritidis*) were suspended in PBS and diluted from 2 × 10^9 bacteria/ml to 2 × 10^4 bacteria/ml, using tenfold dilution steps. 0 05 ml of each suspension was added to the same volume of COA reagents and observed for agglutination as before. 0 05 ml of the same bacterial

TABLE 4 *Co-agglutination (COA) of Lipopolysaccharides (LPS) Extracted from* S paratyphi *A var durazzo,* S typhimurium *SH4809 and* S enteritidis *SH1262*

Reagent containing antiserum against	COA*) of LPS *S paratyphi* A var durazzo conc. μg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}	*S typhimurium* SH4809 conc. μg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}	*S enteritidis* SH1262 conc. μg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
PM BSA	2+	+	2+	1+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AM-BSA	–	–	–	–	–	–	2+	2+	2+	1+	–	–	–	–	–	–	–	–
TM BSA	–	–	–	–	–	–	–	–	–	–	–	–	2+	2+	2+	1+	–	–

a) The agglutination was not as clear as when testing whole bacteria and did not appear until after 90 seconds

TABLE 2 *Co-agglutination (COA) Compared with Standard Slide Agglutination (SSA) of Known Salmonella Bacteria. For the Co-agglutination Test Staphylococci Sensitized with Anti PM BSA Anti AM BSA and Anti TM BSA Sera were Used For the Standard Slide Agglutination Test Salmonella O Factor Sera 2 4 and 9 were Used*

Salmonella serogroup	No of strains	No positive in COA[a] with anti												No positive in SSA[a] with sera										
		PM BSA				AM BSA				TM BSA				O2				O4				O9		
		E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg	Dc	E	BA	Bg
A (O1,2 12)	24	24	24	24	24	0				0				22	8	23	9	0				0		
B (O1 4,5 12)	25	0				25	25	25	25	0				0				25	25	25	25	0		
C (O6 7 6,8,8,20)	25	0				1				0				0				0				0		
D (O1 9 12)	25	0				0				25	25	25	22	0				0				25	25	25
E (O3 10,3 15 3,34 1,3 19)	25	0				0				0				0				0				0		

[a]) 2 + reactions

E = endo-agar Bg = brilliant green-phenol red agar
BA = blood agar base Dc = desoxycholate-citrate agar

No entry = reaction not determined

12), C (O6 7 6 8 8 20), D (O1 9 12) and E (O3 10 3 15 3 34; 1 3 19). Each strain was grown on four different substrates, blood agar base (BA), endo-agar desoxycholate-citrate agar (Dc-agar) and brilliant green-phenol red agar (Bg-agar) In COA, all serogroups A, B and D bacteria gave a strong and direct agglutination (within seconds) with the homologous reagent, irrespective of whether the bacteria were grown on BA, endo-agar or Bg-agar (Table 2) When colonies from Dc-agar were tested, the agglutination was more difficult to record but was still positive. None of the strains gave a positive reaction with the heterologous reagent (tested only with colonies grown on endo-agar) Strains representing serogroups C and E did not cause agglutination, with one exception. One of 14 strains belonging to serogroup C2 agglutinated with the anti AM BSA sensitized reagent (Table 2). However the agglutination could not be seen until after 1 min.

When SSA with factor O2 O4 and O9 sera were used, the reactions were comparable with COA as regards bacteria belonging to serogroups B and D (Table 2) Of the serogroup A bacteria grown on endo-agar 22 out of 24 agglutinated in the factor O2 serum, the agglutination was relatively weak, although visible to the naked eye. In many instances no agglutination was seen with colonies grown on the other substrates. No agglutination occurred when bacteria belonging to serogroups C and E were tested (Table 2).

Co-agglutination of Unknown Bacteria from Faecal Samples

The applicability of the COA method for typing *Salmonella* bacteria was subsequently investigated by testing unknown enteric bacteria from fresh faecal samples. Most of the bacterial colonies tested were taken from Bg-agar or endo-agar A total of 316 bacterial strains were tested and also identified by conventional methods. All 94 *Salmonella* serogroup B strains and 14 serogroup D strains gave a strong and direct agglutination in homologous reagents (Table 3). No false positive reactions were observed. Out of 184 strains representing *Salmonella* serogroups C1 (O6 7), C2 (O6 8) E1 (O3 10), E4 (O1 3 19), G2 (O1 13 23), K (O18) and L (O21) positive reaction was observed with one strain belonging to serogroup C2 with the anti AM BSA sensitized reagent. No positive reactions were recorded among the 24 non-*Salmonella* strains (Table 3). *Strains belonging to the genus Proteus* dominated in this group No *Salmonella* serogroup

TABLE 3. *Co-agglutination (COA) of Suspect* Salmonella *Bacteria*

Serogroup	No. of strains	No. of positive in COA*) with anti PM BSA	AM BSA	TM BSA
B (O1 4,5,12) 15 species	94	0	94	0
C1 (O6 7) 1 species	22	0	0	0
C2 (O6,8) 4 species	130	0	1	0
D1 (O1 9 12) 4 species	14	0	0	14
E1 (O3 10) 5 species	20	0	0	0
E4 (O1,3 19) 2 species	9	0	0	0
G2 (O1 13,23) 1 species	1	0	0	0
K (O18) 1 species	1	0	0	0
L (O21) 1 species	1	0	0	0
Non Salmonella	24	0	0	0
Total	316	0	95	14

*) 2 reactions were recorded

A bacteria were found among the samples examined.

Co-agglutination of Lipopolysaccharides from Salmonella *Bacteria*

One advantage of the COA method as compared to SSA is the ability to detect soluble antigens (20 25). Lipopolysaccharides (LPS) extracted from *S paratyphi* A var durazzo (O2, 12_1 12_3), *S typhimurium* SH4809 (O4 5 12_2) and *S enteritidis* SH1262 (O9 12_2) were suspended in PBS in tenfold dilution steps in concentrations ranging from 10^3 µg/ml to 10^{-2}µg/ml. 0 05 ml of each suspension was added to 0.05 ml of COA reagents as before. Agglutination in homologous reagents occurred in concentrations from 1 µg/ml and upwards. No agglutination was seen when lipopolysaccharides in concentrations of up to 1000 µg/ml were tested against heterologous reagents (Table 4). No agglutination appeared when unsensitized staphylococci were used. It should be stressed that the agglutination reactions were evident but not as strong as when whole bacteria were tested. Furthermore, the agglutination did not appear until after 90 seconds, although undiluted serum was used to sensitize the staphylococci.

Sensitivity of the Co-agglutination Method Compared with that of Indirect Immunofluorescence

A further investigation was carried out for the purpose of comparing the sensitivity of the COA method with that of indirect immunofluorescence. Overnight cultures in nutrient broth (Difco, Bacto no 3) of the indicator bacteria (*S paratyphi* A, *S typhimurium* and *S enteritidis*) were suspended in PBS and diluted from 2 × 10^9 bacteria/ml to 2 × 10^4 bacteria/ml, using tenfold dilution steps. 0 05 ml of each suspension was added to the same volume of COA reagent and observed for agglutination as before. 0 05 ml of the same bacterial

TABLE 4 *Co-agglutination (COA) of Lipopolysaccharides (LPS) Extracted from* S paratyphi *A var durazzo* S typhimurium *SH4809 and* S enteritidis *SH1262*

Reagent containing antiserum against	COA*) of LPS: *S paratyphi* A var durazzo, conc. µg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}	*S typhimurium* SH4809, conc. µg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}	*S enteritidis* SH1262, conc. µg/ml 10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
PM BSA	2+	2+	2+	1+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AM-BSA	–	–	–	–	–	–	2+	2+	2+	1+	–	–	–	–	–	–	–	–
TM-BSA	–	–	–	–	–	–	–	–	–	–	–	–	2+	2+	2+	1+	–	–

*) The agglutination was not as clear as when testing whole bacteria and did not appear until after 90 seconds.

TABLE 5 *Sensitivity of Co-agglutination (COA) Compared with Indirect Immunofluorescence (IFL) S paratyphi A S.typhimurium and S.enteritidis Tested against Homologous Reagents (Anti PM BSA Anti AM BSA and Anti TM BSA Sera Respectively)*

No. bacteria/ 0 05 ml PBS	COA of S.paratyphi A	COA of S.typhimurium	COA of S.enteritidis	IFL[a] on S paratyphi A	IFL[a] on S typhimurium	IFL[a] on S enteritidis
10^8	2 +	2 +	2 +	positive	positive	positive
10^7	–	–	–	positive	positive	positive
10^6	–	–	–	positive	positive	positive
10^5	–	–	–	positive	positive	positive
10^4	–	–	–	negative	negative	negative
10^3	–	–	–	negative	negative	negative

[a]) Positive reaction = at least 10 bacteria/50 visual fields.

suspension was placed on a glass slide for IFL examination. The bacteria were tested only against homologous reagents, since the specificity was well established.

With the COA method, the reactions were positive only with the highest concentration of bacteria ($2 \times 10^9/ml = 10^8$ bacteria in the test) In IFL, positive reactions (defined as at least 10 bacteria/50 visual fields) could be seen when using a concentration of 10^5 bacteria/0 05 ml PBS (Table 5). Thus with the criteria used, the IFL method was at least 1000 times more sensitive than the COA method for identification of *Salmonella* bacteria.

DISCUSSION

Sensitization of *Staphylococcus aureus* strain Cowan I with antisera against PM BSA (O-antigen 2) AM BSA (O-antigen 4) and TM BSA (O-antigen 9) resulted in excellent diagnostic reagents for detection of bacteria belonging to *Salmonella* serogroups A, B and D respectively (Tables 1–3). With the dilutions used, the agglutination reactions appeared within few seconds and were strong. This was true irrespective of the substrate from which the colonies were taken (Table 2)

The SSA method, using factor O2 serum and *S paratyphi* A bacteria, was positive when the colonies were taken from endo- and Bg-agar only Colonies grown on Dc agar and BA often failed to give agglutination The agglutination was moreover relatively weak. The reason for this was probably that the factor O2 antibody concentration used was too low Problems in the preparation of high titre factor O2 antiserum using *S paratyphi* A bacteria as immunogen have often been observed. However the PM BSA antigen gave high titre antisera.

The specificity of the COA method was high (Tables 2 and 3). This is a consequence of the high specificity of the sera raised against the synthetic disaccharide-protein conjugates, as demonstrated previously (4 11 18 26 27). However two of 144 *Salmonella serogroup C2 strains* examined reacted with the anti AM BSA sensitized staphylococci (Tables 2 and 3). The reason for this cross-reactivity is unclear The antiserum is specific for the abequose 1→3 mannose disaccharide (representing O-antigen 4 determinant) whereas the serogroup C2 strains have an abequose 1→3 rhamnose disaccharide (probably representing O-antigen 8 determinant) in their O polysaccharide chain (15 17) This structural difference should be sufficient not to allow an O8 antigen AM BSA antibody interaction.

The COA method required rather small quantities of PM BSA, AM BSA and TM BSA antibodies. The final dilution in the reagent was 1/1000 In SSA, O factor antisera are often used in dilutions ranging from 1/5 to 1/50 It is evident that the use of COA can result in a substantial saving of antiserum.

For a positive COA reaction, approximately 10^8 bacteria had to be mixed with the reagent. Such a concentration of bacteria is readily found in colony material. When the COA method was used with soluble O-antigens in the form of the micellar aggregate found in water soluble phenol-water extracted lipopolysaccharides, approximately 0 05 µg LPS gave a positive reaction. This amount is equivalent to approximately 10^7 bacteria. It must be stressed however that when using soluble O-antigen, the COA reaction is more difficult to register than when using whole bacteria. We therefore consider it unlikely that COA could be used for detection of soluble O-antigens in e g. faecal samples

As expected, indirect immunofluorescence (IFL) was much more sensitive than COA for the

detection of *Salmonella* bacteria (Table 5). When 10^5 bacteria were placed on the slide, at least 10 bacteria/50 visual fields could be seen. This implies that the sensitivity of IFL is higher and that, dependent on the criteria established, lower concentrations of bacteria can be detected. The higher sensitivity of IFL as compared to COA must be weighed against the fact that COA can be performed more rapidly and does not require any sophisticated equipment.

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CHLAMYDIA PSITTACI INFECTION IN DANISH CATTLE

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Rønsholt, L. *Chlamydia psittaci* infection in Danish cattle. Acta path. microbiol. scand. Sect. B, 86: 291–297, 1978.

Intestinal tract infection by Chlamydia psittaci was demonstrated in one cattle herd by isolation from faecal specimens, using embryonated eggs. Such infections were observed in all animals younger than 12 months, in 60% of the heifers and in none of the adult cows. The presence of infection correlated (r = 0.511) with the serum titre of complement fixation antibodies against chlamydial antigen. Young calves, which were spontaneously infected with *Chlamydia* postnatally, developed diarrhea and moderate interstitial pneumonia. The results of histological sections and isolation of the agent from tissue specimens indicated *Chlamydia* to be the cause of these conditions. The strain isolated (*ROS* DK/KVL-6/BJ) was identified as *C. psittaci*. The morphology of the organism and it's pathogenicity in guinea-pigs were studied. In embryonated eggs, a dose response curve was demonstrated for the *ROS* strain, which differed in that respect from another member of this species tested, viz. *EBA* (59-795).

Key words: *Chlamydia psittaci*, intestinal tract infection, pathogenicity, cattle, isolation, serology.

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Chlamydia is an obligatory intracellular parasite belonging to the order Chlamydiales (28). The agents are divided into two subspecies, viz. *Chlamydia trachomatis* and *Chlamydia psittaci* (18). In man, the former may cause trachoma, inclusion conjunctivitis, lymphogranuloma venereum and other genital tract infections (5). Although *C. psittaci* is responsible for the zoonoses of ornithosis, this species mainly causes infections in animals. A great number of animal species may be infected, resulting in different types of infection (25). In cattle, *Chlamydia* may cause epizootic bovine abortion (EBA), seminal vesiculitis (SVS), sporadic bovine encephalomyelitis (SBE), polyarthritis, pneumonia and persistent intestinal infection (22, 23, 25).

Chlamydia possess a common group-specific antigen of lipopolysaccharide character, which is active in the complement fixation test (CFT) (3). *C. trachomatis* may be subdivided into 15 serotypes (30), whereas only two distinct types have been identified for *C. psittaci*. Serotype 2 is connected with SBE and polyarthritis in calves, while the agents of the remaining recognized diseases in cattle have been associated with type 1 (24). However, future investigations will probably unmask more serotypes within *C. psittaci*.

Zoonotic infections caused by *C. psittaci* have been reported in Denmark (34), as has also the isolation of these agents from imported psittacines and Danish pigeons (9, 21). Except for suspected sporadic cases of chlamydial-induced bovine abortions (22), no connection between disease and chlamydial infection has been reported in Danish cattle, though up to 19% of serum specimens from these adult animals contain complement fixation (CF) antibodies to *Chlamydia*. The percentage of positive sera increased proportionally when younger animals were included in the test (23).

Since the first isolation of *Chlamydia* from persistent intestinal infection in calves by *York &*

Baker in the U.S.A. in 1951 (36) similar findings in both calves and cattle have been reported from several other countries (25).

The intention of the present investigation was to study whether Danish cattle were subject to intestinal infection by *Chlamydia* to examine their distribution within one herd and, if possible, to connect such infections with clinical and pathological findings.

MATERIAL AND METHODS

Animals. Seventeen animals of different ages (Table 1) were selected at random from a milking-herd (Rostrup Jutland) consisting of 103 heifers and cows, including 11% of animals with CF antibodies to *Chlamydia* (23). In addition, included in the investigation were two 10-day-old conventionally reared Jersey twin-calves (E 1 & 2) which had been transferred to the herd. Neither the dam nor these calves had CF antibodies to *Chlamydia*.

Isolation and identification of Chlamydia. Before transfer of the two twin-calves, 5 g faeces was collected from the rectum for isolation purposes. Three weeks later a similar sample was collected from all 19 animals, after which the transferred twins were sacrificed and autopsied. Five g lung tissue from the left and right apical pulmonal lobi and 5 g of the ileum wall were carefully washed in phosphate buffered saline (PBS pH 7 2), and tested. The samples were triturated under aseptic precautions and treated according to the procedure described by *Page et al* (20). The final supernatant of a triple centrifugation was inoculated as a single dosis of 0 2 ml into the yolk-sac of five 7-day-old embryonated eggs. The yolk-sacs of two eggs were harvested 7 days post infection (p.i.) and passed on for two further passages. The remaining eggs in each passage were followed for up to 19 days p.i. unless death occurred earlier.

The presence of *Chlamydia* was indicated by impression smears from yolk-sac specimens, stained according to *Gimenez* (10) which showed typical detached red elementary bodies of uniform size, while CF antigen (Ag) afforded the proof (see below). Isolates were checked for mycoplasma (31) and contamination by bacteria, using 5% blood-agar plates incubated aerobically and in pyrogallol bags. Embryonated eggs inoculated with PBS and yolk-sac material from non-infected eggs were used as controls.

Complement fixation tests. Aether-extracted and acetone-precipitated group-specific Ag (35), prepared from yolk sacs of embryonated eggs which died 7–10 days p.i. in the fourth passage, was tested in CFT against a known positive serum (B 577/type 1 kindly provided by Professor *J. Storz*, Colorado State university). Furthermore, Ag from the corresponding *C. psittaci* strain (*EBA* 59-795) isolated from an American outbreak of epizootic bovine abortion (27), and an aether-extracted *C. trachomatis* CF Ag (kindly provided by Dr C. H. Mordhost, Statens Seruminstitut, Copenhagen) of a strain (*TRIC E*/DK/SS-1/ON) isolated from a case of inclusion conjunctivitis in a Danish child (16), were included in serological cross-reaction tests.

The test was performed according to the procedure described previously (23). More than 75% complement fixation by the test Ag Ab complex was considered to be positive.

Purification of Chlamydia. Infected yolk-sacs were homogenized, trypsinized and submitted to differential centrifugation combined with the addition of Celite* (Sigma, gr III D 5384) (19). Finally the sediment was suspended in 0 5 ml PBS (pH 7 0) with an admixture of 0 2 M sucrose per gram yolk-sac.

Growth in cell culture. Nearly confluent monolayers of HeLa cells in Leighton-tubes were incubated for 3 h at 37°C during frequent agitation with 0 6 ml partly purified agents with a titre of 3 × $10^{4.0}$ chicken embryo 50% lethal doses ($CELD_{50}$). Controltubes were treated in the same way with partly purified yolk-sac of non-infected eggs. The cultures were examined for formation of inclusions by staining according to Gimenez (10) and Giemsa (13), and for glucogen-containing inclusions according to *Gordon & Quan* (12) 24 h, 44 h, 68 h and 92 h p.i.

Hyperimmune serum. Rabbits were immunized with 0 5 ml partly purified agents, correspooding to 1 × $10^{6.8-8.3}$ $CELD_{50}$, emulsified in incomplete Freunds adjuvant by simultaneous multiple intracutaneous inoculations, followed by intramuscular inoculation of the agent without adjuvant 3 weeks later. Optimal immune response was obtained 4 weeks after the first immunization (22).

Histological examination. Formalin-fixed sections of lung and ileum tissue were stained with haematoxylin-eosin. Cryostate sections of ileum were stained according to Gimenez (10).

Dose-response curve in embryonated eggs. The growth in 7-day-old embryonated eggs of a representative chlamydial strain (*ROS* DK/KVL 6/B3) isolated from faeces of a heifer without clinical symptoms from the herd in question (Table 1 B 3), was compared with the *EBA* strain by estimating average day of death p.i. (ADD) related to a tenfold dilution of agents (11). Twenty eggs were inoculated with 0 2 ml of each of the dilutions and candled at the same time once a day.

Although there might have been a possibility of different *Chlamydia* strains being mixed when handled through many subpassages over a long period, the fact that the isolated strains preserved their characteristics during such proceedings seemed to indicate that cross contamination was avoided by the precautions taken.

Pathogenicity on guinea pigs. Two adults guinea-pigs were inoculated intraperitoneally with 0 2 ml (1 × $10^{5.8}$ $CELD_{50}$) of the partly purified *ROS* strain. Control animals consisted of two guinea pegs from the same flock inoculated with the carrier-substance for the agent.

Sulphonamide sensitivity. To a partly purified suspension of the *ROS* strain with a titre of 1 × $10^{4.8}$ $CELD_{50}$, was added 1 mg of sulphadiazine per 0 2 ml. The suspension was left on the bench for half-an-hour before being titrated in embryonated eggs. Similar titrations of the *Chlamydia* suspension were also performed without sulphonamide admixture.

Ultrastructural investigation. Segments of yolk-sac membranes from embryonated eggs inoculated with the EM and ROS strains were fixed in 2.5% glutaraldehyde for 2 h, followed by 1% osmium tetroxide overnight at 4°C. The sections were embedded in Vestopal W® cut on a LKB ultratome I and examined in a JEOL JEM 100 B electron microscope.

RESULTS

Isolation studies. Agents were isolated from faeces of cattle from the herd in question. More shedders were found among young animals than among older ones (Table 1). As estimated by ADD of inoculated embryonated eggs, the degree of infection was inversely proportional to age and occurred most frequently in the youngest calves. The two 10-day-old calves (E 1 & 2, Table 1) developed intestinal infection with *Chlamydia* during their three-week stay in the herd.

Complement fixation tests. Stationary CF titres were observed even over longer periods in most of the animals (Table 1). Only one 6 month-old calf (D 3) demonstrated a significant increase in titre from 1:10 to 1:40. Discrepancy existed between the immunological findings and the intestinal isolation results in some cases, such as seropositive animals which apparently did not house the agent (A2, B1), or seronegative animals from which *Chlamydia* could be isolated (E1 & 2). On the other hand, most CF positive animals, except for the one-month-old calves, were found among the young animals which all harboured the agent. Correlation existed between the CF titre and the severity of intestinal infection, especially when the findings in calves E 1 and E 2 were excluded ($r = 0.511$).

The introduced twin-calves. The animals (E 1 & 2 Table 1) had periodic diarrhoea during the period of observation. At the end of this period the clinical findings were normal, apart from a reduced nutritional status. The only change seen at autopsy was a hyperplastic thickening of the ileum wall. Histology revealed hyperplastic ileitis with reticulo-endothelial hyperplasia of Peyer's patches, focally dilated lacteals and submucous lymph vessels and infiltration with eosinophiles, neutrophiles, plasma cells and proliferation of macrophages (Fig. 1). Sections of the apical lobes from the lungs of one

TABLE 1 *Distribution of Faecal Chlamydial Isolates in Different Age Groups of One Cattle Herd. Serum Titres against Chlamydia in Complement Fixation Test (CFT) are given relative to the Day of Faecal Sampling (Day 0)*

		Isolates[a]	Mean ADD	Positive %	Reciprocal[b] values of serum dilutions positive against Chlamydia −64	−30	−21	0	+35 days
Cows	A1	–				–		–	5
	2	–			20	20		20	20
	3	–						–	5
	4	–		0	10	10		10	10
Heifers	B1	–						40	40
	2	+						20	20
	3	+						10	5
	4	–						–	–
	5	+	20	60	10	20		20	40
Calves aged about 12 months	C1	+ +						40	40
	2	+						10	10
	3	+ +						20	20
	4	+	18	100				20	
Calves aged about 6 months	D1	+						40	40
	2	+ + +						20	20
	3	+						10	40
	4	+ + +	15	100				40	80
Calves[c] aged 1 month	E1	+ + +					–	–	
	2	+ +	9	100			–	–	

[a] Isolates from faeces graduated according to »Average Day of Death« (ADD) post infection in embryonated eggs (ADD inversely proportional to titre of agents): + + + = ADD ≤ 14; + + = ADD 15–18 incl.; + = ADD ≥ 19; – = no lethal effect. Three experiments were undertaken.

[b] = not tested; – = negative in CFT

[c] Transferred twin-calves, which initially when 10 days old, did not excrete *Chlamydia*.

Baker in the U.S.A. in 1951 (36) similar findings in both calves and cattle have been reported from several other countries (25)

The intention of the present investigation was to study whether Danish cattle were subject to intestinal infection by *Chlamydia* to examine their distribution within one herd and, if possible, to connect such infections with clinical and pathological findings.

MATERIAL AND METHODS

Animals Seventeen animals of different ages (Table 1) were selected at random from a milking herd (Rostrup Jutland) consisting of 103 heifers and cows, including 11% of animals with CF antibodies to *Chlamydia* (23) In addition, included in the investigation were two 10-day-old, conventionally reared Jersey twin-calves (E 1 & 2) which had been transferred to the herd. Neither the dam nor these calves had CF antibodies to *Chlamydia*

Isolation and identification of Chlamydia Before transfer of the two twin-calves, 5 g faeces was collected from the rectum for isolation purposes. Three weeks later a similar sample was collected from all 19 animals, after which the transferred twins were sacrificed and autopsied. Five g lung tissue from the left and right apical pulmonal lobi and 5 g of the ileum wall were carefully washed in phosphate buffered saline (PBS, pH 7 2), and tested The samples were triturated under aseptic precautions and treated according to the procedure described by *Page et al* (20). The final supernatant of a triple centrifugation was inoculated as a single dosis of 0 2 ml into the yolk-sac of five 7-day-old embryonated eggs. The yolk-sacs of two eggs were harvested 7 days post infection (p.i.) and passed on for two further passages. The remaining eggs in each passage were followed for up to 19 days p.i unless death occurred earlier

The presence of *Chlamydia* was indicated by impression smears from yolk-sac specimens, stained according to *Gimenez* (10) which showed typical detached red elementary bodies of uniform size, while CF antigen (Ag) afforded the proof (see below) Isolates were checked for mycoplasma (31) and contamination by bacteria, using 5% blood agar plates incubated aerobically and in pyrogallol bags. Embryonated eggs inoculated with PBS and yolk-sac material from non infected eggs were used as controls.

Complement fixation tests Aether-extracted and acetone-precipitated group-specific Ag (35) prepared from yolk-sacs of embryonated eggs which died 7–10 days p.i. in the fourth passage, was tested in CFT against a known positive serum (B 577/type 1 kindly provided by Professor *J Storz* Colorado State university). Furthermore, Ag from the corresponding *C psittaci* strain (*EBA* 59 795), isolated from an American outbreak of epizootic bovine abortion (27), and an aether-extracted *C trachomatis* CF Ag (kindly provided by Dr C H Mordhost. Statens Serumsinstitut, Copenhagen) of a strain (*TRIC E/DK/SS-1/ON*) isolated from a case of inclusion conjunctivitis in a Danish child (16), were included in serological cross-reaction tests.

The test was performed according to the procedure described previously (23). More than 75% complement fixation by the test Ag-Ab complex was considered to be positive.

Purification of Chlamydia. Infected yolk-sacs were homogenized, trypsinized and submitted to differential centrifugation combined with the addition of Celite® (Sigma, gr III D 5384) (19). Finally the sediment was suspended in 0.5 ml PBS (pH 7 0) with an admixture of 0 2 M sucrose per gram yolk-sac

Growth in cell culture Nearly confluent monolayers of HeLa cells in Leighton-tubes were incubated for 3 h at 37°C during frequent agitation with 0 6 ml partly purified agents with a titre of $3 \times 10^{6.0}$ chicken embryo 50% lethal doses ($CELD_{50}$). Controltubes were treated in the same way with partly purified yolk-sac of non-infected eggs. The cultures were examined for formation of inclusions by staining according to Gimenez (10) and Giemsa (13), and for glucogen-containing inclusions according to *Gordon & Quan* (12) 24 h, 44 h, 68 h and 92 h p.i.

Hyperimmune serum Rabbits were immunized with 0.5 ml partly purified agents, corresponding to $1 \times 10^{6.8-8.3}$ $CELD_{50}$, emulsified in incomplete Freunds adjuvant by simultaneous multiple intracutaneous inoculations, followed by intramuscular inoculation of the agent without adjuvant 3 weeks later Optimal immune response was obtained 4 weeks after the first immunization (22).

Histological examination. Formalin-fixed sections of lung and ileum tissue were stained with haematoxylin eosin. Cryostate sections of ileum were stained according to Gimenez (10).

Dose-response curve in embryonated eggs The growth in 7-day-old embryonated eggs of a representative chlamydial strain (*ROS* DK/KVL 6/B3) isolated from faeces of a heifer without clinical symptoms from the herd in question (Table 1 B 3), was compared with the *EBA* strain by estimating average day of death p.i (ADD) related to a tenfold dilution of agents (11) Twenty eggs were inoculated with 0 2 ml of each of the dilutions and candled at the same time once a day

Although there might have been a possibility of different *Chlamydia* strains being mixed when handled through many subpassages over a long period, the fact that the isolated strains preserved their characteristics during such proceedings seemed to indicate that cross contamination was avoided by the precautions taken

Pathogenicity on guinea pigs Two adults guinea-pigs were inoculated intraperitoneally with 0 2 ml ($1 \times 10^{5.0}$ $CELD_{50}$) of the partly purified *ROS* strain. Control animals consisted of two guinea-pigs from the same flock inoculated with the carrier-substance for the agent

Sulphonamide sensitivity To a partly purified suspension of the *ROS* strain with a titre of $1 \times 10^{4.0}$ $CELD_{50}$, was added 1 mg of sulphadiazine per 0 2 ml The suspension was left on the bench for half-an-hour before being titrated in embryonated eggs Similar titrations of the *Chlamydia* suspension were also performed without sulphonamide admixture.

TABLE 2. *Serological Cross-Reaction in Complement Fixation Test between Group-Antigen Extracted from One Chlamydia trachomatis and Two Different* Chlamydia psittaci *Strains and Homologous Rabbit Antisera*

Antigen	Homologous antiserum		
	TRIC	EBA	ROS
TRIC E/DK/SS-1/ON	320	320	640
EBA 59-795	320	320	640
ROS DK/KVL-6/B3	160	160	320
Control Ag	–	–	–

organs examined yielded no bacteria after inoculation on blood-agar plates. Examination of related impression smears stained according to Gimenez, showed no chamydial elementary bodies. However isolation trials in eggs were positive already in the first passage, while the same organs from control animals were negative for three successive subpassages. The control animals were clinically healthy and no pathological changes in the organs examined could be demonstrated.

Development of agent in embryonated eggs. Differentiation between the *ROS* and *EBA* strains was demonstrated by the growth characteristics in eggs. The titration endpoints of the *ROS* strain were obtained in shorter time, normally within 8 days p.i., thus resulting in a steeper slope with a coefficient of −2.5 as compared to −1.0 of that of the *EBA* strain (Fig. 3). This characteristic regression coefficient of the dose-response curve was stable during 19 egg-passages and after a 126-day passage in a cow. The optimal harvest of the two strains differed quantitatively since the *EBA* strain constantly produced up to ten times more infectious agents than the *ROS* strain.

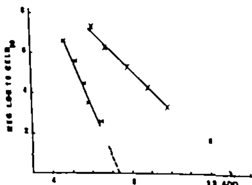


Fig. 3. Dose-response curves for the *Chlamydia* strains *ROS* DK/KVL-6/B3 (R) and *EBA* 59-795 (E), estimated by end-titration and average day of death (ADD) in embryonated eggs.

DISCUSSION

Chlamydia may be considered to be an ubiquitary agent which causes persistent intestinal infection in Danish cattle. As reported previously (23), a relatively high percentage of cattle have CF antibodies to *Chlamydia*. As demonstrated in the present study *C. psittaci* may frequently be isolated from faecal specimens from a clinically normal herd with a representative percentage of seropositive animals.

Studies of the ADD (11) of embryonated eggs inoculated with *Chlamydia* isolated from faeces of the cattle studied, gave reproducible results. Infection occurred early in life. Further more the greatest dissemination of the organism can be seen among the younger animals, as was anticipated from an investigation of chlamydial CF antibodies in the herd, which demonstrated that the highest frequency of seropositive animals was among the 5–12 month-old calves (23). According to the results of isolation (Table 1), the intensity of infection decreased and the infection vanished with increasing age. This agrees well with the observation that *Chlamydia* is isolated only rarely from faeces of apparently healthy cattle more than 2 years of age. (25).

Though an overall correlation between chlamydial intestinal infection and the level of specific CF antibodies was claimed in the present study, a few animals showed deviation. Thus very young calves (E 1 & 2, Table 1) did not develop CF antibodies in spite of severe intestinal *Chlamydia* infection. This does not seem to be explained by local restriction of the organisms to the epithelium (29). Such a lack of Ab development has been reported previously in Chlamydial-infected calves during their first 6–8 weeks of life (4, 26, 32). In this period Ig A of locally accumulated plasma cells may play an important part, since these cells are increased in number and seem to be the only type of phagocytosing cell which destroys the organisms (4). On the other hand, stationary CF titres without demonstrable intestinal infection have been found in older animals (20); a similar observation was made in the present study. This discrepancy might express a persistent infection limited to the phagocytosing cells outside the epithelium. As stated previously by other authors (1, 15), the occurrence of CF antibodies against *Chlamydia* in the present investigation had little, if any, importance in combating the infection, since the antibodies were present long time before and along with high level of demonstrable infection. The role of cell-mediated immuneresponse in this respect is still unsolved, but probably it influences the infection to a greater extent (7, 14).



Fig 1 Hyperplastically changed ileum from a calf with *Chlamydia* infection. The section shows dilation of lacteals, inflammatory cell accumulation in lamina propria and reticulo-endothelial hyperplasia of Peyer's patches. Haematoxylin-eosin, × 100

calf (E 1) showed disseminated interstitial pneumonia with proliferation of alveole macrophages and slight neutrophile infiltration (Fig. 2).

Inoculation of tissue specimens and regional lymph nodes on blood agar plates showed a flora consisting of Gramnegative rods, streptococci and *Streptomyces spp* Lung tissue did not cause any changes in primary calf-kidney monolayer for three successive subpassages, maintained for 7 days each.

Chlamydia was isolated from the ileum wall of both calves (E1 & 2) in a number which was one log unit ($CELD_{50}$) greater than from faeces, estimated by the difference in ADD Furthermore, the agent was demonstrated in the lung tissue of one calf (E 1) in a lower concentration equivalent to 14 ADD in the second egg-passage. In cryostate sections of ileum stained according to Gimenez, focally distributed inclusions were demonstrated in about 1–2% of the epithelial cells. These were most frequently located on the tips of the villi but a few were also seen in the crypts of Lieberkühn, and in cells belonging to lamina propria.

Identification of chlamydial isolates The identity of antigen preparation from four isolates (B2, B3 B5 & E2) passaged four times in embryonated eggs, was confirmed in CFT against the known positive contol sera. Cross-reaction in CFT was demonstrated (Table 2) between the *C trachomatis* strain (TRIC E), the *EBA* strain and the isolated *ROS* strain against homologous convalescent sera. No unspecific reaction against non-infected embryonated egg Ag was observed.

Glucogen-containing inclusions were not observed during the life cycle of the agents in cell culture.

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Inoculation of guinea pigs. Infection by the *ROS* strain resulted in ruffled coat, anorexia and lethargy 4–5 days p.i. Fibrinous peritonitis, disseminated liver necrosis and splenomegaly were found. The



Fig 2 Section from apical lobus of one calf (E 1) with *Chlamydia* infection, showing disseminated interstitial pneumonia dominated by proliferation of alveole macrophages. Haematoxylin-eosin, × 250

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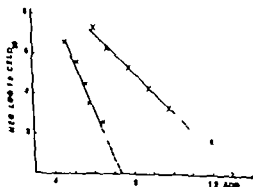


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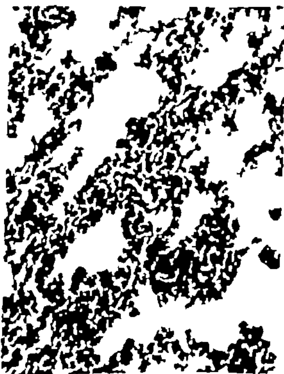


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As chlamydial infections are ubiquitary in cattle, and no species-specific antibodies have been detected in convalescent sera, the CF test generally cannot be employed as a diagnostic tool, even in severe infections. The clinical picture of the two youngest spontaneously infected calves (*E 1 & 2*) was not different from that of other calves of the same age in the herd. Benign pneumonia, diagnosed by clinical observations, was often found in these calves. However in this respect the herd studied did not differ from what is normally observed in cattle herds in general. *Chlamydia* infection may therefore contribute to disease in Danish calves to a greater extent than hitherto recognized. The ileum seemed to be an important target organ for *Chlamydia*. The histological findings in sections of this organ from animals in the present study resembled those found in chronic ileal infections of calves experimentally infected with the more virulent polyarthritis strain (6) The latter experiment demonstrated further that a chlamydial infection of the mucosal epithelial cells was able to penetrate to the lymph vessels via macrophages (4 6) Similar generalization of the *ROS* strain might also take place in the present investigation. However whether the pulmonal lesions were part of a systemic infection, or were caused by inhalation of *Chlamydia* could not be established. However interstitial pneumonia has been reported previously to follow oral infection with a chlamydial strain known to cause intestinal infection (26).

The intestinal strain isolated, *ROS* DK/KVL-6/B3 was identified as a *Chlamydia* agent by serology Its inability to form glucogen-containing inclusions and its non-susceptibility to sulphonamide identified it further as *C psittaci* (18). Morphologically the development forms of the *ROS* strain were identical with those reported from other *C psittaci* strains such as the *6 BC* strain (2).

The *ROS* strain and other faecal strains of cattle are supposed to belong to *C psittaci* serotype 1 as, for instance, the *EBA* strain (24). Like different isolated intestinal strains described in previous reports (36) besides strains of serotype 2 (17), the *ROS* strain isolated also showed pathogenicity for the guinea-pig However this is not a property of the *EBA* strain (17). Furthermore, the stable dose response curve in embryonated eggs distinguished the *ROS* strain from the *EBA* strain. These observations seem to support previous proposals (8 33) for a further subdivision of *C psittaci* into more types.

The question if a persistent intestinal infection by *Chlamydia* can cause other clinical affections in cattle is still open. Neither did the present investigation solve this problem, although experimentally it has been shown that, in addition to pneumonia, the *ROS* strain is able to cause mastitis and placentitis (22).

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As chlamydial infections are ubiquitary in cattle, and no species-specific antibodies have been detected in convalescent sera, the CF test generally cannot be employed as a diagnostic tool even in severe infections. The clinical picture of the two youngest spontaneously infected calves (E 1 & 2) was not different from that of other calves of the same age in the herd. Benign pneumonia, diagnosed by clinical observations, was often found in these calves. However in this respect the herd studied did not differ from what is normally observed in cattle herds in general *Chlamydia* infection may therefore contribute to disease in Danish calves to a greater extent than hitherto recognized. The ileum seemed to be an important target organ for *Chlamydia* The histological findings in sections of this organ from animals in the present study resembled those found in chronic ileal infections of calves experimentally infected with the more virulent polyarthritis strain (6) The latter experiment demonstrated further that a chlamydial infection of the mucosal epithelial cells was able to penetrate to the lymph vessels via macrophages (4 6) Similar generalization of the *ROS* strain might also take place in the present investigation. However whether the pulmonal lesions were part of a systemic infection, or were caused by inhalation of *Chlamydia* could not be established. However interstitial pneumonia has been reported previously to follow oral infection with a chlamydial strain known to cause intestinal infection (26).

The intestinal strain isolated, *ROS* DK/KVL-6/B3 was identified as a *Chlamydia* agent by serology Its inability to form glucogen-containing inclusions and its non-susceptibility to sulphonamide identified it further as *C psittaci* (18) Morphologically the development forms of the *ROS* strain were identical with those reported from other *C psittaci* strains such as the *6 BC* strain (2)

The *ROS* strain and other faecal strains of cattle are supposed to belong to *C psittaci* serotype 1 as, for instance, the *EBA* strain (24). Like different isolated intestinal strains described in previous reports (36) besides strains of serotype 2 (17), the *ROS* strain isolated also showed pathogenicity for the guinea pig. However this is not a property of the *EBA* strain (17). Furthermore, the stable dose-response curve in embryonated eggs distinguished the *ROS* strain from the *EBA* strain. These observations seem to support previous proposals (8 33) for a further subdivision of *C psittaci* into more types.

The question if a persistent intestinal infection by *Chlamydia* can cause other clinical affections in cattle is still open. Neither did the present investigation solve this problem, although experimentally it has been shown that, in addition to pneumonia, the *ROS* strain is able to cause mastitis and placentitis (22).

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EXPERIMENTAL »RUNDE« VIRUS INFECTIONS IN EMBRYONATED EGGS AND CHICKENS

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Three-day-old chicks and 11–12 day embryonated eggs were inoculated with 100 BMLD50 (baby mouse lethal doses) of Runde virus. Chicks were infected subcutaneously and eggs in the allantoic or amniotic cavities, in the yolk sac, or on the chorioallantoic membrane. »Runde« virus produced viraemia and antibody responses in 3-day-old chicks. The virus multiplied only in the amniotic cavity of the embryonated eggs and was detected in the brains of the embryos from day 5–9 p.i. Out of five eggs left to hatch, two hatched on time, while in three unhatched eggs the chicks were alive but extremely weak. Virus was detected in the brains of all five chicks, and high antibody titres were found in the two which hatched. These two chicks had »epilepsy-like« attacks. The results suggested that one passage in chicks or eggs reduced the mouse pathogenicity of »Runde« virus. No antigenic difference between chick- and mouse-passaged virus could be demonstrated by gel precipitation.

Key words: »Corona-like«, bird arbovirus, embryonic infection, CNS disease.

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»Runde« virus was isolated from unengorged *Ixodes uriae* ticks collected in the seabird colonies at Runde, Norway, in 1973 (3, 4). Two strains were isolated which showed a morphology resembling corona virus, and antibodies were demonstrated in host-birds for *I. uriae*. The ecological circumstances pointed to a previously unrecognized arbovirus circulating between seabirds and ticks. Subsequently Finnish workers found a comparable virus in *I. uriae* ticks collected at Røst in the Lofoten Islands, Norway (2).

The ability of »Runde« virus to produce persistent infections in cell cultures and mice has already been established and studied to some extent (6). However, since the principal vertebrate hosts for »Runde« virus seem to be birds, it was considered reasonable to study the ability of »Runde« virus to infect the available *in vivo* avian experimental models, viz. chickens and embryonated fowl eggs.

MATERIALS AND METHODS

The isolation history of virus strain Ru E81 has been given in detail elsewhere (4). The virus was used after two passages in newborn mouse brains, followed by four passages in BHK 21/c13 cells as reported previously (4). The infected medium from the 4th passage was used as virus inoculum, and media from uninfected BHK 21/c13 cultures were used as control. Virus doses were calculated by the formula of *Reed & Muench* (1) based on titrations in newborn mice, and were recorded as $BMLD_{50}$ (baby mouse lethal doses).

For serological identification of virus and antibody determinations, haemagglutination inhibition test (HAI) and immunoelectroosmophoresis (IEOP) were used, as described previously (4, 5).

RESULTS

Experiment 1

Ten 3-day-old chickens were inoculated with 100 $BMLD_{50}$ subcutaneously. Blood specimens for detection of viraemia or anamnestic antibody

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TABLE 1 *Experimental Infection of Chickens with 100 $BMLD_{50}$ of »Runde« Virus Subcutaneously*

Chicken no	Days after infection 2	3	5	10	20
1	2.8[a]	ND[b]	ND	40[c]	320
2	–	–	–	10	80
3	Dead virus+[d]				
4	2 6	ND	ND	20	320
5	–	3 5	ND	20	640
6	2.6	Dead, virus+[e]			
7	–	2 8	ND	20	320
8	3 1	ND	ND	40	320
9	–	–	–	20	160
10	–	–	–	10	80

[a] *Viraemia in $\log_{10}$ $BMLD_{50}$/0 02 ml,– less than 1 5 logs.*
[b] Not done
[c] Antibody titre in HAI (haemagglutination inhibition)
[d] Virus detected in blood and in brain; not titrated.
Virus detected in blood only

response were taken on days 2 3 5 10 and 20 post inoculation (p.i).

Viraemia (≥ 1 5 $\log_{10}$ $BMLD_{50}$) was detected in seven chickens, and increases in antibody were found in all survivors. The results are shown in Table 1

One of the chickens was found dead on day 2 p.i. and another died during bleeding on day 3 In the former virus was detected in both blood and brain in the latter in the blood only The last eight chickens were killed on day 25 p i No virus was detected in a pooled sample from the brains or in pooled blood from day 10 p.i

Experiment 2

We wanted to find out whether »Runde« virus multiplied in embryonated eggs.

Twenty 12-day-old eggs were inoculated with 100 $BMLD_{50}$ in the allantoic, amniotic or yolk sac cavities and on the chorio-allantoic membrane. Four eggs were given virus and one control medium per inoculation route. Eggs were harvested for virus determination on days 2 3 4 and 5 p.i.

Virus was detected in the amniotic fluid only on all days of sampling. Quantification of virus was impossible by baby mouse brain inoculation. The mice showed paralysis and paresis to varying extents, the symptoms starting on day 8–9 but all survived until day 14 when they were killed. »Runde« virus was detected readily in the brain suspensions from these mice by HAI and IEOP

Experiment 3

Thirty 11-day-old embryonated eggs were inoculated with 100 $BMLD_{50}$ in the amniotic sac. Five eggs were harvested to test the embryos for virus on days 5 to 9 p.i. while the last five were left for hatching

The brains of the embryos were pooled according to day of harvest. Suspensions were made by known methods (4) and were inoculated intracerebrally into newborn mice, using three litters of eight mice per suspension

Again, the mice showed paresis and paralysis to varying extents but survived until they were killed on day 14 p.i. The effects were the same, irrespective of the age of the chick embryos. »Runde« virus was identified readily by HAI and IEOP in the inoculated mouse brains, and also directly in the chicken brain suspensions.

Two of the remaining five eggs hatched on time. The last three did not show any signs of hatching during a subsequent observation of eight hours, after which they were opened. The chickens were still alive but extremely weak, and did not react at all to handling They were killed at once by exsanguination.

The two survivors were handled and fed in the same way as other chickens kept at the Department of Laboratory Animals, National Institute of Public Health They would feed normally and also otherwise seem healthy for varying periods (minutes to hours) but suddenly without any notice would collapse in tonic-clonic spasms. During these episodes, they did not seem to be conscious of, or at least did not react to handling and otherwise frightening disturbances in the environment. The attacks would last for approximately 1–3 minutes and stop just as suddenly as they had started. The chickens often started feeding again the moment they were back on their feet. After showing the same symptoms for eight days, they were killed by exsanguination.

The brains of these five chickens were treated individually and suspensions were inoculated intra cerebrally into newborn mouse litters and also on BHK 21/c13 cell cultures. Virus was detected and identified in all chicken brains but in none of the blood specimens Virus was recovered in mice as well as in cell cultures, but could not be quantified by mouse LD_{50} or cell CPE.

In the sera of the two surviving chickens, high antibody titres to »Runde« virus (640 1280) were demonstrated by HAI

Due to the reduced pathogenicity for baby mice of virus isolated from eggs and chickens, we tried to detect antigenic differences between mouse-passaged and chicken-passaged virus, employing the Ouchter

bay modification CHI (closed hexagon immunodiffusion) as reported previously (4 7) Reactions of identity were found between mouse-passaged and chicken-passaged virus by this technique

DISCUSSION

These experiments have shown that »Runde« virus can infect chickens and embryonated eggs, and that the virus persisted in the embryos during the last 10 days of development and in the brains of the chickens after hatching. It has also been shown that »Runde« virus infection of embryonated eggs may affect the viability and hatching ability and probably produces CNS disease in the chickens. Host modification of virus pathogenicity was suggested by the prolonged disease produced by one avian passage of an originally mouse-lethal virus strain.

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[c] Antibody titre in HAI (haemagglutination inhibition).
[d] Virus detected in blood and in brain, not titrated
[e] Virus detected in blood only

response were taken on days 2 3 5 10 and 20 post-inoculation (p.i.).

Viraemia ($\geq$ 1 5 $\log_{10}$ $BMLD_{50}$) was detected in seven chickens, and increases in antibody were found in all survivors. The results are shown in Table 1

One of the chickens was found dead on day 2 p.i. and another died during bleeding on day 3 In the former virus was detected in both blood and brain, in the latter in the blood only The last eight chickens were killed on day 25 p.i No virus was detected in a pooled sample from the brains or in pooled blood from day 10 p.i.

Experiment 2

We wanted to find out whether »Runde« virus multiplied in embryonated eggs.

Twenty 12-day-old eggs were inoculated with 100 $BMLD_{50}$ in the allantoic, amniotic or yolk sac cavities and on the chorio-allantoic membrane. Four eggs were given virus and one control medium per inoculation route. Eggs were harvested for virus determination on days 2, 3 4 and 5 p.i.

Virus was detected in the amniotic fluid only on all days of sampling. Quantification of virus was impossible by baby mouse brain inoculation. The mice showed paralysis and paresis to varying extents, the symptoms starting on day 8–9 but all survived until day 14 when they were killed »Runde« virus was detected readily in the brain suspensions from these mice by HAI and IEOP

Experiment 3

Thirty 11-day-old embryonated eggs were inoculated with 100 $BMLD_{50}$ in the amniotic sac. Five eggs were harvested to test the embryos for virus on days 5 to 9 p.i. while the last five were left for hatching.

The brains of the embryos were pooled according to day of harvest. Suspensions were made by known methods (4) and were inoculated intracerebrally into newborn mice, using three litters of eight mice per suspension.

Again, the mice showed paresis and paralysis to varying extents but survived until they were killed on day 14 p.i. The effects were the same, irrespective of the age of the chick embryos. »Runde« virus was identified readily by HAI and IEOP in the inoculated mouse brains, and also directly in the chicken brain suspensions.

Two of the remaining five eggs hatched on time. The last three did not show any signs of hatching during a subsequent observation of eight hours, after which they were opened. The chickens were still alive but extremely weak, and did not react at all to handling They were killed at once by exsanguination.

The two survivors were handled and fed in the same way as other chickens kept at the Department of Laboratory Animals, National Institute of Public Health. They would feed normally and also otherwise seem healthy for varying periods (minutes to hours) but suddenly without any notice would collapse in tonic-clonic spasms. During these episodes, they did not seem to be conscious of or at least did not react to handling and otherwise frightening disturbances in the environment. The attacks would last for approximately 1–3 minutes and stop just as suddenly as they had started The chickens often started feeding again the moment they were back on their feet After showing the same symptoms for eight days, they were killed by exsanguination.

The brains of these five chickens were treated individually and suspensions were inoculated intracerebrally into newborn mouse litters and also on BHK 21/c13 cell cultures. Virus was detected and identified in all chicken brains but in none of the blood specimens. Virus was recovered in mice as well as in cell cultures, but could not be quantified by mouse LD_{50} or cell CPE.

In the sera of the two surviving chickens, high antibody titres to »Runde« virus (640 1280) were demonstrated by HAI

Due to the reduced pathogenicity for baby mice of virus isolated from eggs and chickens, we tried to detect antigenic differences between mouse-passaged and chicken-passaged virus, employing the Ouchter

lary modification CHI (closed hexagon immunodiffusion) as reported previously (4 7). Reactions of identity were found between mouse-passaged and chicken-passaged virus by this technique.

DISCUSSION

These experiments have shown that »Runde« virus can infect chickens and embryonated eggs, and that the virus persisted in the embryos during the last 10 days of development and in the brains of the chickens after hatching. It has also been shown that »Runde« virus infection of embryonated eggs may affect the viability and hatching ability and probably produces CNS disease in the chickens. Host modification of virus pathogenicity was suggested by the prolonged disease produced by one avian passage of an originally mouse-lethal virus strain.

The author is grateful to *Einar Bruerold* and *Hallgrim Sægrov* for expert technical assistance.

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Acta path. microbiol. scand. Sect. B, 86: 303–307, 1978

EFFECT OF HUMAN LEUKOCYTE INTERFERON ON THE PERMEABILITY OF THE CYTOPLASMA MEMBRANE OF CULTURED CELLS

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Degré, M. Effect of human leukocyte interferon on the permeability of the cytoplasma membrane of cultured cells. Acta path. microbiol. scand. Sect. B, 86: 303–307, 1978.

The effect of human leukocyte interferon on the cytoplasma membrane of cultured homologous cells has been investigated. U-amnion cells were labelled with (1-^{14}C) alpha-aminoisobutyric acid (AIB), a low-molecular-weight non-metabolizable amino acid. Following uptake, the AIB is released spontaneously from the cells. Treatment of cells with interferon reduced the AIB release. This effect was dosedependent, was neutralized by anti-interferon serum and was strongly reduced by trypsin treatment.

Key words: interferon; cytoplasma membrane.

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An effect on the cytoplasma membrane is probably a crucially important part of the action of interferon on homologous cells. It has been reported by several groups of investigators that in some virus-cell systems interferon inhibits a late stage in virus production, assembly, maturation or release of virus particles (1, 2, 11, 15, 16). While the intracellular markers of e.g. murine leukaemia virus are produced normally, complete virus particles are released in reduced numbers from interferon-treated cells (11). It has been suggested that this might be a result of an interferon-induced alteration of cell membrane physiology.

To investigate the interferon effect on the stability of the cell membrane, we employed ^{3}H labelled uridine as a low-molecular-weight cytoplasmic marker (10). Treatment of human U-amnion cells with low concentrations of human leukocyte interferon reduced the spontaneous release of ^{3}H uridine from the cells, thus supporting the theory that interferon treatment stabilizes the cell membrane. Since the publication of that report, an improvement of this method has been described by *Thelestam & Möllby* (17), using (1-^{14}C) alpha aminoisobutyric acid (AIB). This compound is a low-molecular weight (mol.wt. 103) non-metabolizable amino acid, and is therefore a good cytoplasmic marker. It has been shown that the sensitivity of the assay for membrane stability is high.

In the present study we have investigated the effect of human interferon on the spontaneous release of AIB from cultured homologous cells.

MATERIALS AND METHODS

Cells

The U line of human amnion cells was originally received from Dr. *K. Cantell*, Helsinki. The cells were grown in Eagle's minimal essential medium (MEM, GIBCO) with the addition of 5 per cent inactivated calf serum, $NaHCO_3$ and antibiotics. The cells were maintained in the same medium with 2 per cent calf serum.

Human embryonic lung cells (HEL) were prepared in this laboratory. They were grown in 50 per cent Eagle's MEM and 50 per cent medium 199 (GIBCO), with the addition of 10 per cent calf serum, $NaHCO_3$ and

antibiotics. The serum concentration was reduced to 5 and later to 2 per cent during maintenance. The HEL cells were in their 5th to 15th passage during the present study

Virus

Vesicular stomatitis virus (VSV), Indiana strain, was grown in L–F_1 mouse fibroblast cells. Samples were stored at −70° C and a fresh ampoule was used for each experiment. Infectivity titres were assayed by the infectivity endpoint micromethod in L–F_1 and U cells.

Interferon

Human leukocyte interferon preparations were kindly provided by Dr *K Cantell*, Helsinki. Both crude interferon and partially purified preparations (4) were employed. The results obtained with the different preparations were identical and therefore are not presented separately. Mock interferon preparations were also obtained from Dr *K Cantell*. Mock interferons were prepared in the same way as the leukocyte interferons, except for omission of the Sendai virus induction of the leukocytes.

Anti interferon Serum

Rabbit anti-human globulin against leukocyte interferon was received from the Antiviral Substances Program National Institute of Allergy and Infectious Diseases, Bethesda, Md

Assay of the Biological Effect of Interferon

Antiviral activity of interferon preparations was tested by the infectivity inhibition micromethod employing VSV and HEL cells. Details of the test are described elsewhere (8). Titres are expressed in international standard units related to the 69/19 standard

Interferon effect on cell growth was tested as described in detail elsewhere (9). Briefly 1 ml of medium containing interferon dilutions was added to freshly seeded U cells, 10^5 cells per tube. At least five tubes were used for each dilution. Control tubes were seeded with medium without interferon. The cells were incubated stationary in 5 per cent CO_2 atmosphere at 37° C. After incubation for 3 days the monolayers were trypsinized and the cells were counted in a haemocytometer. Viability was more than 95 per cent, as determined by the trypan blue staining method

Interferon Effect on Release of AIB

(1–14C) alpha-aminoisobutyric acid (AIB) was purchased from The Radio-Chemical Centre, Amersham. Stock solutions made in Hanks BS were kept at −20° C. Tubes were seeded with 10^5 U cells each in MEM. Three-day-old monolayers were incubated with AIB diluted in MEM to contain 1 µCi per ml. After incubation for 1 hour the cells were washed three times with fresh medium to remove any radioactivity not incorporated into the cells. Interferon diluted in MEM was then added, using 5–8 tubes for each dilution. MEM alone was added to the control tubes. After incubation at 37° C for various times, 0.1 ml of the supernatant was transferred to a scintillation vial containing 10 ml Aerosol. Samples were counted in a Packard liquid scintillator for 10 minutes. The activity in the control culture supernatants was considered as 100 per cent, and the interferon treated cultures were related to the controls.

In some experiments, the remaining medium was decanted and the cells were suspended in 0.9 ml fresh MEM and 0.1 ml of the non-ionic detergent Triton-X 100 at a final concentration of 0.25 per cent (v/v). The tubes were incubated for 15 minutes and agitated in a Vortex mixer. This treatment causes complete lysis of the cytoplasma membrane of the cells, thus releasing the incorporated radioactivity into the medium (18). After lysis, 0.1 ml was transferred to a scintillation vial containing Aerosol and the radioactivity was determined as described above. The total radioactivity taken up by the cells was equal to the sum of released and cell-bound radioactivity

RESULTS

Spontaneous Release of AIB from Normal U Cells

Three-day-old monolayers of U cells were labelled with AIB as described in Materials and Methods. The amounts of extra-cellular and cell-bound radioactivity were determined after various periods of incubation. Results of a representative experiment are shown in Fig. 1. After uptake, some of the radioactivity was released spontaneously from the labelled cells into the medium. The total radioactivity i.e. the sum of extracellular and cellbound radioactivity was constant during the time of the experiment. The kinetics of spontaneous release were similar in several repeated experiments.

Interferon Effect on Spontaneous Release of AIB from Normal U Cells

200 units interferon per ml diluted in MEM were added to the cells immediately after removal of the radioactive AIB-containing medium. Control tubes were supplemented with MEM only. After incuba

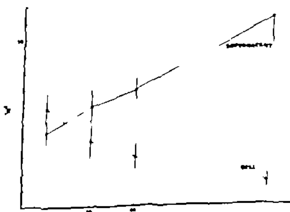


Fig 1 Spontaneous release of AIB from U-amnion cells. Vertical bars indicate standard deviation (SD)

tion for 15, 30, 45 and 90 minutes, 0.1 ml was removed from the supernatant and the radioactivity was determined (Fig. 2). The supernatant of the interferon treated cultures contained less radioactivity after incubation for 45 and 90 minutes than the supernatant of the control cultures.

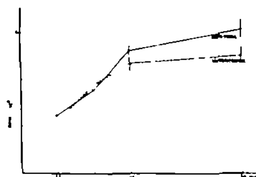


Fig. 2. Effect of human leukocyte interferon on the spontaneous release of AIB from U amnion cells. Vertical bars indicate standard deviation (SD).

The interferon effect on the AIB release was dose dependent (Table 1). A slight increase (105–115 per cent of the controls) was seen regularly after treatment with very low concentrations of interferon. Higher concentrations of interferon (200 units per ml or more) reduced the extracellular radioactivity.

TABLE 1 *Effect of Various Concentrations of Interferon on the Spontaneous Release of AIB from U Cells*

Interferon Units per ml	Counts per minute ± SD	Percentage of control
0	801 ± 41	
5	832 ± 55	104
10	764 ± 67	95
50	764 ± 82	95
200	739 ± 70	92
1000	674 ± 54	84

AIB release was measured after incubation for 90 minutes.

Specificity of the Suppressing Effect of Interferon

The specificity of the interferon effect was tested by exposing the interferon to treatment with physico-chemical factors known to have well-defined effects on the biological activities of interferon. Trypsin treatment is known to eliminate the antiviral effect, while heating at 56° C for 30 minutes has no major influence. In addition, specific antiserum is known to neuralize both the antiviral

TABLE 2 *Specificity of Interferon Effect on the Cell Membrane Permeability*

	Antiviral titre	Number of cells × 10,000 ± SD (% of controls)	Release of AIB % of total [b] radioactivity
Experiment I			
Control cells		41 ± 8.2	78
Interferon	1584	22 ± 4.5 (56)	58
Interferon treated at 56° C for 30 min.	1000	21 ± 3.2 (51)	53
Interferon treated with trypsin	125	37 ± 9.9 (90)	68
Experiment II			
Control cells		21 ± 3.3	38
Interferon	160	11 ± 0.9 (52)	29
Mock interferon	<10	18 ± 4.5 (85)	39
Interferon treated with antiinterferon globulin	<10	20 ± 3.9 (95)	39

Number of cells after incubation for 3 days with the test substance.
[b] AIB release was measured after incubation for 90 minutes.
Interferon was incubated with anti-interferon globulin overnight.

antibiotics. The serum concentration was reduced to 5 and later to 2 per cent during maintenance. The HEL cells were in their 5th to 15th passage during the present study

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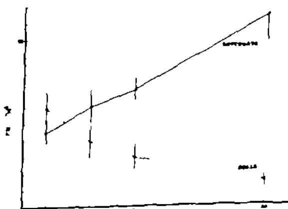


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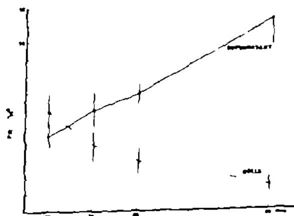


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and non-antiviral activities of interferon. Following treatment of interferon, samples were tested for effect on AIB release. Antiviral activity and effect on cell growth were determined in parallel experiments (Table 2) 1500 units interferon per ml reduced the AIB release by 24 per cent and the cell growth by 44 per cent. Heating had little if any effect on the activities, while trypsin treatment strongly reduced all three activites. Anti interferon globulin had no effect by itself and had no influence on the effect of the low interferon concentrations (1–5 units) on the AIB release. When provoked by 200 units interferon, all three activities were neutralized by treatment with anti-interferon globulin. Mock interferon showed no significant activity when tested by the same three parameters.

DISCUSSION

AIB was chosen as a cytoplasmic marker mainly because it is not metabolized in the cell (7). Therefore its release from the cells must be primarily dependent on the state of the cytoplasma membrane. The release of AIB is also dependent on the solvent in which the cells are incubated. Eagle's MEM was chosen for the present experiments because the spontaneous release into this medium is more pronounced than into other solutions (17). Therefore a possible reduction of the release by stabilization of cell membrane could be more easily demonstrated. The extent of spontaneous release of AIB from U amnion cells was comparable to that found by others (17) from human embryonal fibroblast cells.

The present results show that human leukocyte interferon inhibits the spontaneous release of a non metabolizable cytoplasmic marker from the homologous U amnion cells. This effects was dose dependent and correlated to the antiviral and cell growth inhibitory activities of the interferon preparation. Furthermore, the effect seems to be specific and is eliminated by trypsin treatment and anti-interferon treatment of interferon preparation. This finding confirms the earlier results obtained with ^{3}H-uridine as cytoplasmic marker (10) The findings indicate that the reduction of release is not specific for the one marker used in the earlier experiments

There is considerable experimental evidence suggesting that interferon treatment causes modifications of the cell surface. Murine leukaemia cells produced a reduced number of complete extracellular virus particles, while several intracellular markers were not influenced (11) A marked accumulation of virus particles at the surface indicated that the blocking effect is effective at a late stage of virus production, probably connected with the release process (5). Signs of altered surface characteristics were also observed in various cells without the presence of virus infection. Such alterations were registered by a number of different parameters, such as expression of histocompatibility antigens (14), altered binding of radioactive concanavalin A (12), cholera toxin and tyreoideastimulating hormone (TSH) (13) to the cell surface, effect on the transport of thymidine (3), effect on the spontaneous release of uridine (10), alteration of the density of membrane and presence of intramembrane granules (6). The present findings are compatible with these observations and confirm that interferons alter the cytoplasma membrane.

The quantitative aspects of the present study differ somewhat from our earlier findings, since larger concentrations of interferon were necessary to reduce the release of AIB than that of ^{3}H-uridine (10). Since the AIB release can be considered as a marker of cytoplasma membrane permeability the difference might suggest that intracellular events involving uridine metabolism might be influenced by the interferon treatment.

Very low concentrations of interferon (1–5 units per ml) in several experiments stimulated slightly AIB release from the cells. However this effect was not neutralized by the specific antiserum, and therefore should probably be considered as non-specific.

AIB was originally introduced in order to study the effect of cytolytic substance on the membrane permeability (17). The present results indicate that this substance may also be applied to the study of opposite effects i.e. stabilization of cell membrane.

The excellent technical assistance of *Zdenka Krajči* is gratefully acknowledged.

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DETERMINATION OF GENOME SIZE AND BASE RATIO ON DEOXYRIBONUCLEIC ACID FROM MYCOBACTERIA

I. BAESS and B. MANSA

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The genome size and the base ratio of deoxyribonucleic acid (DNA) from 15 different species of mycobacteria have been determined. The molecular weight of the sheared DNA specimens has been estimated.

Key words: Deoxyribonucleic acid, mycobacterium, genome size, base ratio, molecular weight.

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The $C_0t_{1/2}$, i.e. the concentration of a DNA in moles of nucleotides per litre multiplied by the time in seconds required for 50 per cent reassociation, is dependent on the complexity of a DNA. For DNA samples of equal molecular weight and without repeated sequences, the $C_0t_{1/2}$ is proportional to the genome size. It is possible, therefore, to measure the genome size of a bacterium by comparing the $C_0t_{1/2}$ of its DNA with the $C_0t_{1/2}$ of a DNA with a known genome size (7–17).

The base ratio of a DNA can be determined by means of thermal denaturation, since there is correlation between the temperature for denaturation and the guanine (G) plus cytosine (C) content (percentage GC) (14).

In the present investigations, the genome size and the base ratio of 15 mycobacterial species were determined in order to confirm their mutual genetic relationship. Both virulent and non-virulent mycobacteria were included in the study.

MATERIAL AND METHODS

The DNA was isolated and purified from contaminating polysaccharides as described earlier (1). Shearing of DNA at 20,000 p.s.i. in a French pressure cell press and the techniques used for determination of the $C_0t_{1/2}$ have been described previously (2). The $C_0t_{1/2}$ of the mycobacterial DNA samples was compared with the $C_0t_{1/2}$ of DNA of *E. coli* B which has the genome size 2.8 × 10^9 daltons (3–8). Thus, the genome size of each mycobacterial DNA was calculated from the following equation: Genome size = 2.8 × 10^9 × $C_0t_{1/2}$ of mycobacterial DNA/$C_0t_{1/2}$ of *E. coli* DNA. No correction was made for the difference in the GC content of *E. coli* and mycobacteria because there are divergent opinions concerning the influence of the base ratio on the rate of reassociation (9–26).

Analytical ultracentrifugation. The sedimentation velocity of the sheared DNA preparations was determined in a Spinco model E analytical ultracentrifuge equipped with ultraviolet optics. A cell with 12 mm Kel-F centre-piece was used. All analyses were carried out at 25° C and 60,000 rev/min. Exposures were taken at 8-minute

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intervals. The positions of the DNA boundary in the cell were determined by direct scanning of the negatives in an Analytrol (Spinco) from which a 4 5 times enlarged scanning diagram was obtained. The sedimentation coefficient (S_{25}) was calculated from the slope of the line of log r_n versus t_n (where r_n is the distance from the centre of rotation at time t_n). The sedimentation coefficients were corrected for density and viscosity of the solvent used, *i e* 0 15 M sodium chloride, 0 015 M trisodium citrate, *pH* 7 0 (SSC), and 0 556 was used as the value of the partial specific volume ($\bar{v}$) for the sodium salt of DNA (11).

Determination of the base ratio of the DNA was performed by thermal denaturation in 0 1 SSC using the technique described previously (1). The formula GC = (T_m − 53 9) 2 44 as given by *Mandel & Marmur* (14) for the relationship between T_m and GC percentage for DNA in 0 1 SSC was used for the calculations of the GC percentages.

RESULTS AND DISCUSSION

The sedimentation coefficient

$$(S^{0}_{25,w})$$

for the mycobacterial DNA preparations was determined after shearing One group 10 samples, was analysed at a concentration corresponding to an

$$E^{1\,cm}_{260\,nm} = 1\,8 - 2.0\,(\sim 90 - 100\ \mu g/ml)$$

and the remaining *38* samples at an

$$E^{1\,cm}_{260\,nm} = 0\,9 - 1\,0\,(\sim 45 - 50\ \mu g/ml).$$

The preparations represented DNA isolated from the 19 strains listed in Table 1 The sedimentation *coefficient at zero concentration*

$$(S^{0}_{25\,w})$$

was calculated by extrapolation of the reciprocal of the sedimentation values versus concentration. In this way a value of

$$S^{0}_{25\,w} = 8\,68\,S$$

was obtained for all the centrifugations performed. The standard error of this value was calculated to be 0 15 S By insertion into the formula

$$S^{0}_{25\,w} - 2.7 = 0\,01517\,(\text{mol.wt.})^{0\,445}$$

(10), a mean molecular weight (mol.wt.) of 681 000 daltons (S E. = 34 000) was determined for all the sheared, double-stranded DNA samples. The variation in molecular weight (± 5 per cent) is quite

TABLE 1 *Genome Size and Base Ratio of DNA from Mycobacteria*

Bacterial strain	Number of determinations of $C_0t_{1/2}$	Genome size daltons × 10^{-9}	(S.D.)	G + C percentage
M tuberculosis H 37 Rv	16	3 13	(0 15)	66.3
M tuberculosis (wild strain)	4	3 01	(0 05)	66.4
M bovis (wild strain)	6	3 02	(0 32)	66.3
M bovis BGC	10	3 08	(0 11)	66.1
M kansasii ATCC 12478	4	4 29	(0 24)	67.5
M marinum ATCC 927	4	4 51	(0 16)	66.5
M gordonae ATCC 14470	4	4 57	(0.30)	66 1
M avium, serotype II ATCC 25291	20	3 92	(0 20)	70.3
M avium, serotype III (wild strain)	8	4 00	(0 16)	70.5
M intracellulare ATCC 25169	11	4 29	(0 25)	70.0
M scrofulaceum, ATCC 19073	11	4 57	(0 26)	70.2
M xenopi, SSC 989	12	3 29	(0.18)	68 2
M gastri ATCC 25158	4	4 20	(0 14)	66 6
M smegmatis, ATCC 607	36	5 20	(0.36)	69 1
M smegmatis No. 108	4	5 55	(0 35)	69 4
M phlei ATCC 27086	4	4 30	(0 24)	71 4
M fortuitum ATCC 9820	4	4 16	(0 14)	67 5
M farcinogenes, *var senegalense* NCTC 10956	4	4 27	(0.17)	68 0
M farcinogenes, *var tchadense* IEMVT 93 B	4	4 40	(0.22)	

small. A correlation between this variation and that of the mean value of the corresponding four determinations of $C_0t_{\frac{1}{2}}$ could not be demonstrated.

The results of the determination of the genome size of 15 species of mycobacteria and their DNA base ratios are shown in Table 1. From 4 to 36 determinations of $C_0t_{\frac{1}{2}}$ were carried out on DNA from the species, as indicated in the table. It can be seen that the genome sizes of the DNA of the two strains of *M. tuberculosis*, the strain of *M. bovis* and the strain of *M. bovis* BCG are all equal. These and an *M. xenopi* strain, among which the mycobacteria pathogenic for man are found, have smaller genome sizes (3.0–3.3 × 10^9) than the other strains examined, all of which showed values between 3.9 and 5.6 × 10^9 daltons. The genome size of nine of the species included in this investigation has also been determined by *Bradley* (5, 6). If 2.8 × 10^9 daltons had been used for the genome size of *E. coli* in *Bradley*'s calculations, six of the results would have been similar to ours. However, for the three remaining species, *M. avium*, *M. intracellulare* and *M. fortuitum*, the values determined by *Bradley* would still be smaller. UV-absorbing contaminants can result in a false increase in $C_0t_{\frac{1}{2}}$ values and consequently in too high genome sizes. The purity of our DNA specimens has been described in previous publications (1, 2). All DNA's used were of a similar quality as determined by the ratios E_{260}/E_{280} and E_{260}/E_{230}. In some of our specimens the DNA concentration was measured at 260 nm and also determined by the diphenylamine reaction. These measurements have shown good agreement. Thus, at present, we have no explanations for the discrepancies between *Bradley*'s and our own results.

The results of the GC percentages are listed as mean values of two determinations for each DNA sample. The variation obtained in this duplicate determination of T_m (the temperature corresponding to the midpoint of the melting curve) was from 0° C to 0.35° C, which corresponds to a variation of 0 to 0.8 in the values calculated for the GC percentage. The hyperchromicity of the samples studied was generally about 35 per cent. Some DNA samples of *M. kansasii* and *M. gastri* were also used for determination of T_m prior to purification for contaminating polysaccharides. The hyperchromicities of these samples were found to vary between 11 and 23 per cent. However, the GC percentages calculated from them were the same as those found for the purified DNA samples of these species.

Except for *M. gastri*, base ratios have been published previously for all the species reported on here (4, 12, 13, 18, 20, 21, 22, 23, 24, 25). The values presented in these previous reports generally varied up to 9 per cent for the same species, and all except two are lower than the values obtained in the present study. The use of different methods can only partially explain this discrepancy. As regards thermal denaturation, it is essential that the temperature should be measured with an accuracy of at least 0.1° C. It was found that the variation in temperature was considerably more than this when measured at different locations in the cell holder with the cells containing 10 ml of solution. Consequently, in our experiments, the thermistor was placed directly in the stirred DNA solution without interfering with the light beam.

Mandel & Marmur's (14) formula, GC = (T_m − 53.9) 2.44, for DNA in 0.1 SSC, which was used for our calculations is valid on the assumption that the T_m temperatures for DNA with high and low GC content change in a linear fashion with the logarithm of the sodium ion concentration. This has been shown experimentally by *Marmur & Doty* (15) and *Silvestri & Hill* (19). *Owen et al.* (16) studied the T_m of four DNA samples in SSC and a phosphate buffer at several different ionic strengths. These authors found that the slopes of the equations for T_m versus the logarithms of the sodium ion concentration decreased with increasing GC content of the samples. A recalculation of their data using the SSC values exclusively indicated that the slopes did not show any systematic variation. Apparently, further experimental work is needed for a final evaluation of the effect of changes in buffer composition on T_m.

The genome sizes of the mycobacteria studied were all of the same order of magnitude and the GC contents were found to be high, i.e., between 66 and 71 per cent. Furthermore, as mentioned previously, some of the strains have genomes of equal size (~ 3 × 10^9 daltons). Hybridization experiments with DNA from these strains are now in progress in order to examine the homology of their base sequences.

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